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DOCTORAL DISSERTATION

Functional Characterization and Expression of Molluscan Detoxification Enzymes and Transporters Involved in Dietary Allelochemical Resistance

by

Kristen Elizabeth Whalen

June 2008

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and

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FUNCTIONAL CHARACTERIZATION AND EXPRESSION OF MOLLUSCAN DETOXIFICATION ENZYMES AND TRANSPORTERS INVOLVED IN DIETARY ALLELOCHEMICAL RESISTANCE

By

Kristen Elizabeth Whalen

Bachelor of Science, University of North Carolina, Wilmington, 2001

Submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

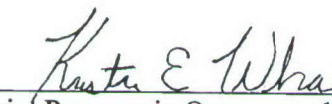
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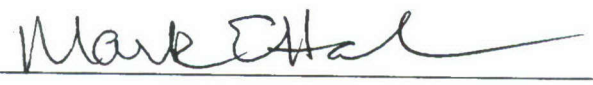
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This thesis is dedicated in loving memory of my sister,
Meredith L. Whalen (1978 – 2001),
for always believing in me

FUNCTIONAL CHARACTERIZATION AND EXPRESSION OF MOLLUSCAN DETOXIFICATION ENZYMES AND TRANSPORTERS INVOLVED IN DIETARY ALLELOCHEMICAL RESISTANCE

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BRIEF ABSTRACT

Understanding how organisms deal with potentially toxic or fitness-reducing allelochemicals is important for understanding patterns of predation and herbivory in the marine environment. The ability of marine consumers to tolerate dietary toxins may involve biochemical resistance mechanisms, which increase the hydrophilicity of compounds and facilitate their active efflux out of sensitive cells and tissues. While several allelochemical-responsive detoxification enzymes have been sequenced and functionally characterized in terrestrial invertebrates feeding on chemically defended host plants, there is virtually no information concerning the role of these biotransformation enzymes that may mediate feeding tolerance in marine invertebrates. The objective of this research was to assess the diversity and dietary regulation of cytochrome P450s (CYP), glutathione *S*-transferases (GST) and ABC transporters in the generalist marine gastropod *Cyphoma gibbosum* feeding on a variety of chemically defended gorgonian corals, and to identify those dietary natural products that act as substrates for these proteins.

Molecular and proteomic techniques identified both allelochemically-responsive CYPs, and constitutively expressed GSTs and transporters in *Cyphoma* digestive glands. Inhibition of *Cyphoma* GST activity by gorgonian extracts and selected allelochemicals (i.e., prostaglandins) indicated that gorgonian diets are likely to contain substrates for molluscan detoxification enzymes. *In vitro* metabolism studies with recombinant CYPs suggested those *Cyphoma* enzymes most closely related to vertebrate fatty acid hydroxylating enzymes may contribute to the detoxification of ichthyodeterrent cyclopentenone prostaglandins found in abundance in selected gorgonian species. Finally, the presence and activity of multixenobiotic resistance transporters in *Cyphoma* and the co-occuring specialist nudibranch, *Tritonia hamnerorum*, suggests these efflux transporters could function as a first line of defense against dietary intoxication. Together, these results suggest marine consumers that regularly exploit allelochemical-rich prey have evolved both general (GST and ABC transporters) and allelochemical-specific (CYP) detoxification mechanisms to tolerate prey chemical defenses.

Thesis Supervisor: Mark E. Hahn
Title: Senior Scientist, Biology Department, WHOI

COMPLETE ABSTRACT

Intense consumer pressure in tropical marine ecosystems not only structures the organization and functioning of marine communities, but has a profound effect on the phenotype of resident organisms. In order to persist in the midst of extreme predation and herbivory, sessile benthic marine organisms, afforded with no means of escape, often produce chemical defenses, termed allelochemicals, which render their tissues unpalatable or toxic to most potential consumers. Despite the fitness-reducing consequences of consuming noxious/toxic prey, less mobile consumers, frequently drawn to their hosts by the chemical refugia they provide, are able to tolerate the toxicological challenges posed by their prey/hosts. The processes underlying dietary allelochemical tolerance are likely mediated, in part, by biochemical resistance mechanisms that have evolved under the selective pressure of host chemical defenses. Although many have invoked this hypothesis to explain the variation in marine consumer tolerance, few studies have examined the enzymatic diversity and corresponding metabolism of dietary allelochemicals.

The shallow Caribbean reefs of the western Atlantic provide an ideal model system with which to investigate marine consumer detoxification and transport-mediated resistance to dietary allelochemicals. The ovulid gastropod *Cyphoma gibbosum* is considered a trophic generalist, feeding upon at least three families of allelochemically-rich gorgonian corals. By virtue of its polyphagous diet, *C. gibbosum* is exposed to a broad and unpredictable array of gorgonian defenses likely necessitating an equally diverse set of counterdefense mechanisms. Using a combination of molecular, proteomic and biochemical approaches, I characterized the diversity and dietary regulation of three families of xenobiotic resistance proteins and examined the potential of gorgonian natural products as substrates for these proteins.

Cloning of multiple cytochrome P450 monooxygenases (CYP4) cDNAs revealed that *Cyphoma* digestive gland tissues expressed a total of twelve new molluscan CYPs grouped within three subfamilies, designated CYP4V10, CYP4BK, and CYP4BL. Expression of CYP4BK and CYP4BL group transcripts was significantly induced in snails feeding on the gorgonian diet *Plexaura homomalla*, (known to contain high concentrations of ichthyodeterrent cyclopentenone prostaglandins), compared to a control diet devoid of gorgonian chemistry. Both CYP4BK and CYP4BL subfamilies form a monophyletic clade sharing extensive sequence identity, and in some cases key active residues, with vertebrate prostaglandin-hydroxylating CYP4A and CYP4F subfamilies, suggesting that allelochemically responsive *Cyphoma* CYP4s may have evolved from an ancestral gene encoding an eicosanoid-metabolizing enzyme. Heterologous expression of select *Cyphoma* CYP4s indicated a possible role in eicosanoid metabolism, providing further evidence of the putative role of *Cyphoma* P450s in the detoxification of dietary prostaglandins and supporting the hypothesis that interactions between *Cyphoma* and its allelochemical-rich prey have contributed to the diversification of some P450 families.

Enzymatic analysis provided evidence for high, constitutive expression of glutathione *S*-transferase (GST) activity in the digestive gland homogenates from snails feeding on all seven allelochemically diverse gorgonian diets. A proteomic approach (HPLC coupled with LC-MS/MS) identified two major GST mu forms and one minor GST theta form – the latter being the first identified from a molluscan source - that were

consistently expressed in the same relative proportion in all snail digestive glands examined. Gorgonian extracts were screened using a bioassay-guided fractionation approach for their ability to inhibit GST activity; all moderately hydrophobic extracts contained putative substrates or inhibitors for *Cyphoma* GSTs, suggesting that the constitutive expression of catalytically versatile enzymes may provide a competitive advantage for generalists that encounter a tremendous diversity of gorgonian defense compounds.

To further determine whether specific gorgonian allelochemicals could be substrates for consumer GSTs, I examined the prostaglandin-mediated inhibition of *Cyphoma* CDNB-conjugating GST activity. Enzymatic analysis indicated that prostaglandin A_2, the series found in the highest concentration (ave. 2% dry weight) in gorgonian tissues, is a competitive inhibitor – and therefore likely a substrate – of affinity-purified *Cyphoma* GSTs. Conservative estimates of the *in vivo* concentration of prostaglandins indicate that GSTs would be operating at or near their physiological capacity in *Cyphoma* digestive gland tissues, and thus may represent effective mechanisms for prostaglandin deactivation and metabolism.

Finally, the presence and function of multixenobiotic resistance (MXR) mechanisms were demonstrated in the generalist *Cyphoma* gibbosum, and a co-occurring specialist nudibranch, *Tritonia hamnerorum*, which consumes a single species of gorgonian coral and sequesters its host's dietary defenses for its own protection. MXR expression was compared between consumers using immunochemical approaches and this analysis revealed that P-glycoprotein was expressed in the nudibranch tissues localized to areas with the highest exposure to gorgonian allelochemicals (i.e., gut lumen and epidermis) - consistent with a role in dietary xenobiotic efflux, while comparable expression was absent in *Cyphoma*. A competitive-based dye efflux assay demonstrated significant MXR-mediated efflux activity in live *Tritonia*, suggesting that the use of transport mechanisms may provide protection against dietary toxins. In the present study, eight partial sequences belonging to both ABCB and ABCC subfamilies were cloned and characterized from *Cyphoma* and *Tritonia* tissues. Predicted protein structure and phylogenetic analyses indicate shared homologies and possible shared function with characterized vertebrate xenobiotic resistance efflux proteins. Quantitative estimates of the expression of the putative glutathione-conjugate efflux protein, MRP-1, indicated constitutive expression of this transcript in the digestive gland of *Cyphoma* individuals feeding on multiple gorgonian diets. The universal expression of MRP transporters in *Cyphoma* digestive tissues may facilitate the efflux of putative glutathione-conjugates of prostaglandin and other lipophilic gorgonian compounds.

The studies described in this thesis provide the most comprehensive investigation thus far of the three phases of detoxification and transport that may protect marine consumers from their chemically defended prey. These data indicate that both general and allelochemical-specific multixenobiotic resistance mechanisms operate in marine consumers to confer resistance to gorgonian allelochemicals. These findings will undoubtedly help provide a framework for understanding the profound variation of consumer tolerance for their chemically rich prey, and more broadly, hold great significance for understanding patterns of predation and herbivory in marine ecosystems.

Acknowledgements

The universe is full of magical things, patiently waiting for our wits to grow sharper.
--Eden Phillpotts

Mount Everest is very high and very cold. Many people are not crazy enough to tackle the icy and desolate cliff faces of such a formidable peak. The journey to the top can be fraught with perilous mishaps that test your resolve and make you wonder why you embarked on the journey in the first place. If that weren't enticing enough, of those who do set out to reach the summit, only one in four will actually make it.

There is a clear parallel here to getting a PhD. It requires persistence, sacrifice and copious amounts of hard work to make it to the finish. It can be a lonely soul-searching endeavor, thankfully punctuated by beautiful moments of grandeur along the way that remind you why it was that you began this epic trek. However, it is the support and encouragement of a great number of people during my journey that has allowed me to reach the summit, and made me the scientist and person I am today.

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Chapter I

Introduction

Background

In marine communities, predation plays a pivotal role in affecting the phenotype of organisms as well as community organization (Duffy & Hay 2001). The intensity of consumer pressure on coral reefs exerts strong selection on prey to avoid being eaten. Prey species unable to physically escape or tolerate consumers often produce noxious compounds, termed allelochemicals, as a mechanism of defense against predation (Paul 1992). Chemically defended prey are often noxious or toxic to most large, mobile consumers, yet appear to be the target of evolutionary opportunity for smaller, more specialized consumers (Hay 1991, Faulkner 1992). However, the physiological and biochemical mechanisms that allow these more specialized marine consumers to tolerate (i.e., consume, manipulate, and/or detoxify) dietary allelochemicals are largely unknown (Sotka & Whalen 2008).

In the field of chemical ecology, rarely have specific adaptive traits been identified down to the biochemical or genetic level (Berenbaum 2002). Examining how chemical interactions at the cellular and molecular level shape the behavior of organisms is paramount in ultimately understanding how chemicals affect the distribution and abundance of organisms in communities. In terrestrial ecology the success of insects is due, in part, to their use of sophisticated detoxification systems to overcome the chemical defenses of their plants (Li et al. 2007). Chemical defenses in general are thought to be an evolutionary driving force behind the development of detoxification genes (Gonzalez & Nebert 1990). Therefore, understanding the role of these genes in allelochemical resistance in marine organisms holds potential significance for understanding patterns of

predation and herbivory in marine systems. While several allelochemical-responsive CYPs and GSTs genes have been sequenced and functionally characterized in insects feeding on chemically defended plants, there is virtually no information concerning the role of these biotransformation enzymes and multixenobiotic transporters in marine invertebrates that also feed on allelochemically-rich prey.

Introduction

Marine Chemical Ecology

The field of marine chemical ecology examines the role chemicals play in mediating a diverse array of interactions among marine organisms. With well over 7,000 natural products, termed secondary metabolites, isolated thus far from marine species (Harper et al. 2001), chemical ecologists are working to elucidate the ecological roles of these compounds in marine systems. Taking cues from their terrestrial counterparts, which had laid the groundwork some thirty years before, marine chemists and ecologists entered into productive collaborations and began exploring how secondary metabolites mediated marine consumer-prey interactions (Pawlik 2000). Research in this field has advanced significantly since the early studies in the 1980's. Laboratory and field based behavioral studies have begun to classify compounds as attractants, protectants or deterrents; however, more recently others have begun to address the physiological and biochemical mechanisms underlying these responses (Targett & Arnold 2001).

Chemical defenses against predation have been a major focus in marine chemical ecology, as evidenced by the number of pertinent reviews (Bakus et al. 1986, Hay &

Fenical 1988, Pawlik et al. 1995, Hay 1996, Paul et al. 2001, Paul & Puglisi 2004). The existence of chemical defenses in marine organisms and their importance in mediating predator-prey and plant-herbivore interactions have been well established. Many sessile benthic invertebrates lack the means to escape consumers and, therefore, have evolved chemical deterrents to ensure their survival (Faulkner & Ghiselin 1983, Faulkner 1992, Stachowicz 2001, O'Neal & Pawlik 2002). Although chemical defenses limit predation and herbivory in tropical marine ecosystems, some consumers still regularly feed on allelochemically rich organisms. The consequences of consuming defensive metabolites can be severe and range from reduced growth and reproductive fitness to decreased survival (Hay 1992, Targett & Arnold 2001). However, for those consumers that consistently feed on chemically defended prey, behavioral adaptations (e.g. avoidance), physiological tolerance (e.g. sequestration, membrane impermeability, rapid excretion) and biochemical resistance mechanisms (e.g. detoxification enzymes) may explain the ability of these consumers to tolerate dietary allelochemicals (Brattsten 1992a). While the first two strategies have been documented in the marine environment (Targett 1979, Targett et al. 1986, Gerhart 1991, Slattery et al. 1998), few studies have investigated natural product detoxification mechanisms in marine invertebrates as a means of coping with noxious dietary compounds.

Gorgonian chemical defenses

Gorgonian corals (Phylum: Cnidaria; Subclass: Octocorallia; Order: Gorgonacea)

are the most conspicuous group of invertebrates on Caribbean coral reefs (Lasker & Coffroth 1983), reaching densities of up to 25 colonies m^{-2} (Goldberg 1973); however, they are consumed by only a limited number of specialized grazers despite their abundance and the intense predation in these ecosystems (O'Neal & Pawlik 2002). Gorgonians, also referred to as octocorals, of the West Indian region (Gulf of Mexico, Antilles, Bahamas, Florida Keys, Bermuda, South America, Caribbean) represent 38% of the known fauna with over 195 species documented (Bayer 1961). The dominance of these invertebrates can be attributed, in part, to the variety of metabolites they produce, collectively known as allelochemicals, which are known potent feeding deterrents (Rodriguez 1995). Numerous feeding studies (Fenical & Pawlik 1991, Pawlik & Fenical 1992, Paul & Puglisi 2004) with crude organic extracts and pure compounds from gorgonians have shown that, with the exception of a few, small mesograzers (Lasker 1985, Lasker et al. 1988, Vreeland & Lasker 1989), more mobile, larger grazers, such as fishes and invertebrates, do not readily consume octocorals. Field-based surveys assessing the palatability of gorgonians found that the majority of lipid-soluble gorgonian extracts deterred reef fish at concentrations an order of magnitude lower than those found in the soft tissues of the corresponding gorgonians (Pawlik et al. 1987).

As of 2001, the subclass Octocorallia accounted for 87% (~1300 compounds) of all reported cnidarian metabolites, with nearly 500 coming from the order Gorgonacea alone (Harper et al. 2001). The majority of these compounds are terpenoids, representing over twenty different skeletal classes with unique substitution patterns and functionalities (Rodriguez 1995). Diterpenoid metabolites (C_{20} compounds) represent the largest

percentage of natural products isolated from gorgonians (~ 73%), while sesquiterpenoids (C_{15} compounds) rank second (14%). Two sesquiterpenes - curcuhydroquinone and curcuquinone - isolated from the octocoral *Pseudopterogorgia rigida,* are extremely active as fish feeding deterrents at low concentrations (Harvell et al. 1988). Similar compounds are well known as secondary metabolites of terrestrial plants and as defensive compounds of insects in terrestrial systems (Aneshansley et al. 1969, Eisner 1970, Thomson 1971). The ubiquity of these terpenoid compounds, coupled with their demonstration of potent feeding deterrence in the marine environment, make them interesting targets for further investigation (Harvell et al. 1988, Coll 1992).

Acetogenins, prostanoids, and highly functionalized steroids have also been reported from gorgonians (Rodriguez 1995). Among the most interesting are the prostanoids, which are produced in large quantities solely in the Caribbean octocoral *Plexaura homomalla* (Gerhart 1984, 1986). Feeding assays in the laboratory and on the reef with prostaglandins isolated from *P. homomalla* (e.g. 15-PGA_2 and their hydrolyzed derivatives), resulted in vomiting or learned aversion to these compounds in fish (Gerhart 1984). Additionally, many interesting sterols have been isolated from gorgonians; however, information on their defensive function is lacking.

Although these chemical defenses limit predation by most potential consumers, some relatively sedentary species such as the ovulid gastropod *Cyphoma gibbosum*, regularly feed on allelochemically rich octocorals (Harvell & Suchanek 1987, Lasker et al. 1988, Ruesink & Harvell 1990, Nowlis 1993). *Cyphoma gibbosum* exclusively feeds on gorgonians and is considered a trophic generalist, because most available species in

several gorgonian families are included in its diet (Lasker et al. 1988). Additionally, it appears that *C. gibbosum*'s tissues do not mirror the chemical composition of its octocoral prey (Cronin et al. 1995), suggesting the possibility that *C. gibbosum* can either efficiently excrete these compounds or biotransform their dietary allelochemicals to less toxic metabolites.

Allelochemical biotransformation and transport

Little is known about the role of allelochemical biotransformation in marine organisms (Kuhajek & Schlenk 2003). In comparison, insect-plant allelochemical interactions are well understood and may provide a conceptual framework for research to elucidate the role of biotransformation enzymes in the chemical ecology of marine invertebrates. In both systems, terrestrial and marine invertebrates must contend with dietary allelochemicals that can have deleterious effects on their growth, reproduction and survival (Feeny 1992). Insects that feed on toxin-containing plants possess biotransformation mechanisms that allow them to exploit a specific subset of chemically defended plants. Berenbaum and colleagues (Berenbaum 1978, Berenbaum & Feeny 1981) were the first to report that xanthotoxin, a linear furanocoumarin produced from umbelliferous plants, was toxic to southern armyworms but harmless to the larvae of black swallowtails. Cohen et al. (Cohen et al. 1989, 1990) later demonstrated that xanthotoxin resistance in swallowtail larvae was conferred by a suite of cytochrome P450 monooxygenases selectively induced upon exposure to xanthotoxin, and responsible for the subsequent oxidation of the furanocoumarin. While the dietary allelochemicals may

be quite different between terrestrial and marine systems, *Cyphoma gibbosum* may have similar detoxification mechanisms to that of insects, allowing this molluscan predator to take advantage of an abundant, energy-rich food source not available to most other consumers likely lacking such biochemical resistance mechanisms. Despite the significance of allelochemicals in the evolution of biotransformation enzymes (Gonzalez & Nebert 1990) and the importance of these enzymes in the detoxification of anthropogenic xenobiotics (Omura 1999), little attention has been paid to the role of biotransformation enzymes in the metabolism of the wealth of dietary allelochemicals from marine organisms.

Biotransformation reactions are just one mechanism by which marine organisms can defend themselves against allelochemicals. A second approach involves multixenobiotic transporters (MXT) that may prevent putative toxins from accumulating in the cell in the first place (Epel 1998). The ability of marine natural products to modulate transporter activity has been assessed only recently using invertebrate models. Several extracts from seaweed and phytoplankton species were found to contain potent substrates/modulators of mussel and sea urchin multixenobiotic transporter activity (Eufemia et al. 2002). Subsequent crude fractionation of the extracts traced the compounds responsible for the inhibition of transporter activity in mussel cells to the moderately hydrophobic fractions. Interestingly, most known substrates for xenobiotic transporters are moderately hydrophobic, amphipathic, low molecular weight, planar molecules (Bard 2000). Exposure to these compounds would most likely occur via ingestion as the mussels actively filter particulate matter from the water column. To combat compound

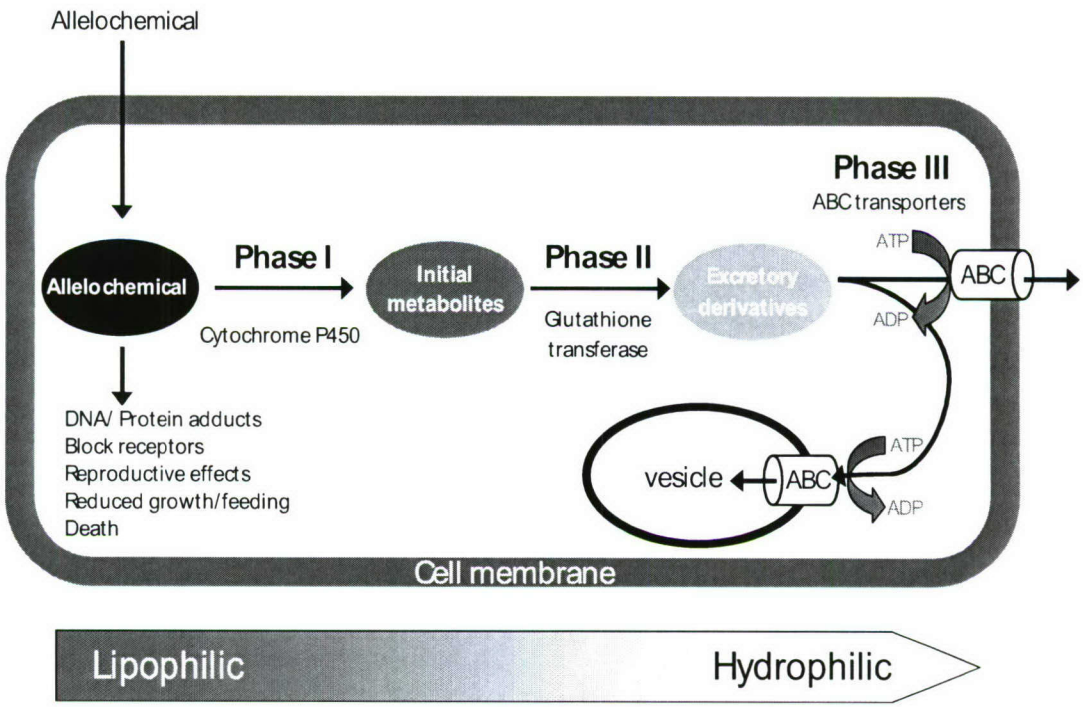
accumulation, MXT are highly expressed in the gills of bivalves (Minier et al. 1993, Smital & Kurelec 1997), one of the primary routes of tissue exposure to dietary compounds. The tissue distribution (e.g. liver, kidney, digestive tract) of these proteins in other marine organisms also suggests a parallel role in protection from diet-derived toxins (Bard 2000). Marine consumers may use similar multixenobiotic transporters to prevent dietary accumulation of noxious gorgonian compounds in concert with other detoxification enzymes.

Much of the diversity of biotransformation enzymes in insects may be the result of co-evolution of prey chemical defenses and predator detoxification mechanisms (Gonzalez & Nebert 1990, Li et al. 2003). I hypothesize that some marine invertebrates may have undergone a similar diversification of detoxification enzymes to combat the continuous barrage of defensive compounds produced by their prey (David et al. 2003). Marine generalists, like *Cyphoma gibbosum*, may have evolved an array of functional enzymes based on different selective forces associated with the biochemical defense profiles of their hosts. Alternatively, selection for resistance to diverse metabolites among marine generalists may have resulted in the development of an enzyme/transporter with broad substrate specificities (Li et al. 2003, Smital et al. 2004). Consequently, understanding the role of allelochemical detoxification and transport in marine organisms will allow us to determine how predators respond biochemically to dietary metabolites, and will contribute to a more general understanding of dietary preferences and patterns of predation on the reef.

Mechanisms of detoxification and transport

The detoxification and excretion of xenobiotics, which can broadly be described by three phases, involve highly complex processes that are able to respond to an organism's chemical environment. Biotransformation is the process by which nonpolar compounds are converted to more water-soluble chemicals that are more easily excreted from the body (Figure 1).

Figure 1. Overview of allelochemical metabolism and transport (reproduced from Sotka & Whalen 2008)



In general, the biotransformation of xenobiotic compounds is accomplished by a limited number of enzymes with broad substrate specificities. The reactions catalyzed by these

enzymes can be divided into phase I and phase II (Williams 1959). Phase III processes involve the energy-dependent transport of multiple structurally and functionally unrelated xenobiotics across a variety of cellular membranes (Litman et al. 2001). Select phase III transport proteins prevent the retention of toxicants in cells and tissues and are complementary to both phase I and II reactions in minimizing xenobiotic toxicity. All three phases have previously been studied in marine invertebrates; however, these studies have focused primarily on their role in the metabolism and transport of anthropogenic compounds (e.g. polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides; reviewed in (Galgani et al. 1996, Eufemia & Epel 2000, Snyder 2000). The participation of detoxification enzymes in the metabolism of dietary allelochemicals in terrestrial systems has been extensively documented (Scott et al. 1998, Li et al. 2007) and I hypothesize that these enzymes may have similar functions in marine organisms.

Phase I – Cytochrome P450

The most familiar phase I reactions involve the addition of a polar functional group (e.g. hydroxyl) onto a compound to increase its hydrophilicity and facilitate excretion. These reactions are accomplished primarily by a suite of inducible enzymes collectively termed cytochrome P450 monooxygenases (CYPs) (Parkinson 2001) found in terrestrial and aquatic organisms ranging from bacteria to vertebrates (Omura 1999). CYPs comprise a large superfamily of heme-thiolate proteins that metabolize a wide range of endogenous and exogenous hydrophobic compounds. Embedded in the

endoplasmic reticulum (ER), the cytochrome P450 enzyme can insert one oxygen atom from molecular oxygen, O_2, onto the substrate as it is held at the active site by lipid interaction. The combination of a relatively non-specific and loose lipid interaction to bind a substrate and an activated oxygen radical to oxidize it makes the reaction mechanism of cytochrome P450 extremely powerful (Brattsten 1992a). In addition to hydroxylation reactions, CYPs catalyze several other types of oxidation reactions including: epoxidation of double bonds, heteroatom (S-, N-, and I-) oxygenation and N-hydroxylation; heteroatom (O-, S-, and N-) dealkylation; ester cleavage; and dehydrogenation (Parkinson 2001).

Each functional gene appears to encode a unique enzyme (Nelson et al. 1993) roughly 500 amino acid residues in length (Omura 1999). Different cytochrome P450 genes are assigned to different families according to their degree of sequence identity (families share > 40% identity; subfamilies share > 55% identity) (Nelson et al. 1993). A comparison of all eukaryotic CYPs found minimal conservation of residues with the exception of amino acids involved in structural conservation in the core of the protein around the heme (Werck-Reichhart & Feyereisen 2000). The heme binding loop, considered to be the "P450 fingerprint region", contains the most characteristic P450 consensus sequence, including the absolutely conserved cysteine that serves as the fifth coordinating ligand to the heme iron. Other conserved sequences include the Glu-X-X-Arg motif in helix K, which participates in core structure stabilization, and the oxygen binding pocket in the I helix involved in oxygen activation and transfer of protons to the active site (Werck-Reichhart & Feyereisen 2000). Microsomal P450 binding and

insertion into the membrane is mediated by a hydrophobic amino-terminal sequence ("signal anchor sequence"), leaving the bulk of the enzyme exposed to the cytoplasmic side of the ER. Adjacent and down stream to the anchor sequence is a proline-rich/basic region that may serve to join the membrane-binding N-terminus to the globular region of the P450 (Graham-Lorence & Peterson 1996). Variable regions throughout the gene are associated with substrate binding and recognition and are often referred to as substrate-recognition sites or SRSs (Werck-Reichhart & Feyereisen 2000). The flexibility of SRS regions allows for structurally variable substrate binding and catalysis.

As of February 2008, there are a total of 7232 known CYP sequences, excluding variants and pseudogenes, representing 781 families (designated by number) (http://drnelson.utmem.edu/CytochromeP450.html). In animals alone, 110 P450 families have been described encompassing 2565 named sequences. The broad substrate specificity and inducible nature of many CYPs are consistent with their proposed central role in feeding habits, since generalist consumers can be expected to encounter a broad array of allelochemicals in their diet. The enormous metabolic diversity of the P450-dependent monooxygenase system provides a comprehensive protective mechanism against a wide variety of xenobiotics (e.g. chemical defenses, hydrocarbons, etc) for organisms challenged with these compounds on a daily basis (Simpson 1997, Mansuy 1998).

For many invertebrates, the true number of P450 genes is unknown. However, sequencing of insect genomes shows extensive proliferation and divergence in both *Drosophila melanogaster* and *Anopheles gambiae* resulting in 90 and 100 annotated P450

genes (http://p450.antibes.inra.fr/) representing 5 families: CYP4, CYP6, CYP9, CYP18 and CYP 28. The extreme diversification of P450 forms and the acquisition of new gene functions are believed to have occurred via gene duplication events (Berenbaum 2002). To fully understand the evolutionary diversification of P450s, research has focused on identifying individual isoforms that metabolize specific dietary compounds (Berenbaum 1983, 1991, Cohen et al. 1992, Petersen et al. 2001), which may have acted as selective agents to promote P450 diversification. For example, furanocoumarin-containing host plants are all but ignored except by a few lepidopterans in the genus *Papilio*. The molecular basis of furanocoumarin resistance in *P. polyxenes* has been attributed to the constitutive expression of CYP6B1v1 and CYP6B1v2, which encode furanocoumarin-metabolizing P450s (Cohen et al. 1992, Ma et al. 1994) and contain a highly conserved furanocoumarin-responsive xenobiotic response element required for basal transcription and inducibility (Petersen et al. 2003). Additionally, specific expression of furanocoumarin-metabolizing CYPs is only detected in larval feeding stages and not in adults, eggs, or pupae, suggesting a function consistent with the dietary metabolism of toxic furanocoumarins (Harrison et al. 2001).

The polyphagous lepidopteran *Helicoverpa zea* also feeds on furanocoumarin-containing plants, but consumes more than 100 different host plant families with vastly different chemistry (Kogan et al. 1978). It has been suggested that this insect generalist can cope with a variety of plant chemical defenses because of the greater diversity, structural flexibility and functional versatility of its CYP6B genes in comparison to the specialist *P. polyxenes* (Li et al. 2004). The cost of increased flexibility, resulting in a

less efficient metabolism of furanocoumarins, is balanced by the acceptance of a wider variety of allelochemicals at the catalytic site. *C. gibbosum*'s CYPs may have similar biochemical traits which may allow this consumer to be an equally successful marine generalist.

Many terpenoids isolated from plants, including monoterpenes from *Eucalyptus* spp., have been shown to limit feeding by herbivores (Boyle et al. 1999). Marsupials that fed exclusively on eucalypt leaves containing the monoterpenes p-cymene and 1,8-cineole use CYP2 and/or CYP3 enzymes to detoxify these natural products (Pass & McLean 2002). Inhibition of 1,8-cineole metabolism by p-cymene metabolites and other plant secondary metabolites may impose a significant constraint on foliage consumption in these generalist marsupials. Inhibitory interactions among dietary compounds may explain obligatory browsing by generalist herbivores. Likewise, *C. gibbosum* feeds on many gorgonians that contain high concentrations of terpenoid compounds, and the production of these compounds is further increased in response to elevated predation by this mollusc (Thornton & Kerr 2002). The mobility of *C. gibbosum* (Birkeland & Gregory 1975) and the variety of gorgonian corals included in its diet may be explained by the need for diet mixing in this species (Harvell & Suchanek 1987). It is believed that consumers are better able to tolerate particular compounds by limiting their residence time on any one species (Snyder et al. 1998). This mollusc may use similar strategies and CYPs to detoxify the range of gorgonian terpenes.

Molluscan Cytochrome P450s

Among eukaryotes the phylum Mollusca ranks second in species diversity after Arthropoda, yet the diversity of molluscan CYP isoforms is poorly understood in comparison to that of insects. This discrepancy results in part from the lack of a fully sequenced molluscan genome, although there are species in the pipeline. Nevertheless, biochemical and molecular studies have provided some information about the diversity of molluscan CYPs.

There is biochemical evidence suggesting that some of the P450s responsible for biotransformation in mammals are present in marine invertebrates in general and molluscs in particular (Snyder 2000). Northern, Southern and Western blot techniques suggest that multiple CYPs (i.e., 1A, 2B, 2E, and 3A-like enzymes) may be expressed in the digestive glands of some molluscs after treatment with prototypical inducers of vertebrate CYPs (Wootton et al. 1995, Wootton et al. 1996, Peters et al. 1998b, a) (Table 1). However, some of this biochemical evidence is equivocal, and the presence of these CYPs has yet to be confirmed with a gene or cDNA sequence.

A few molluscan CYP cDNAs have been cloned and sequenced (Table 1). Both arthropods and molluscs share the CYP4 gene family and in both phyla this family accounts for the majority of known P450s (Rewitz et al. 2006, Strode et al. 2008). To date, multiple CYP4 isoforms have been identified in both freshwater and marine molluscs including *Mytilus galloprovincialis* (Mediterranean mussel), *Haliotis rufescens* (red abalone), *Unio tumidus* (freshwater mussel), *Mytella strigata* (mussel) and *Perna viridis* (Asian green mussel) (Snyder 1998, Chaty et al. 2004). A CYP4 sequence

identified from *U. tumidus* shares high amino acid sequence identity with CYP4C sequences in lobster and *Anopheles* (59-61%) and CYP4V sequences in mouse and trout (54% identity) (Chaty et al. 2004). The ubiquity of CYP4 genes in mammals, bony fishes and invertebrates (Nelson 1998) suggests this gene family arose at least 1.25 billion years ago (Simpson 1997). While substantial information is lacking as to the physiological role and substrate specificity of these CYP4 enzymes in marine invertebrate systems, studies

Table 1. Biochemical and molecular evidence for molluscan CYPs

Proposed CYP Classification[a]	Species	Evidence	Literature or GenBank Reference
CYP1A	*Crassostrea gigas*	Sequence	Boutet et al. 2004
	Mytilus edulis	Western blot	Peters et al. 1998a, Shaw et al. 2000
	Mytilus galloprovincialis	Western blot	Livingstone et al. 1997, Canova et al. 1998, Peters et al. 1998a,b
	Mytilus edulis	Northern blot	Wootton et al. 1995, Wootton et al. 1996
CYP2	*Crassostrea rhizophorae*	Sequence	Rebelo & Moreira 2003
	Mytilus edulis	Western blot	Jonsson et al. 2004
CYP2B	*Mytilus galloprovincialis*	Western blot	Peters et al. 1998a
CYP2E	*Mytilus edulis*	Western blot	Shaw et al. 2000
	Mytilus galloprovincialis	Western blot	Peters et al. 1998b
CYP3A	*Cryptochiton stelleri*	Western blot	DeBusk et al. 2000
	Mytilus edulis	Southern	Wootton et al. 1995
	Mytilus galloprovincialis	Western blot	Peters et al. 1998a
CYP4	*Mytella strigata*	Sequence	GenBank # DQ310714
	Mytilus edulis	Western blot	Jonsson et al. 2004
	Perna viridis	Sequence	GenBank # EU429566
	Unio tumidus	Sequence	Chaty et al. 2004
CYP4A	*Mytilus edulis*	Northern blot	Wootton et al. 1995
	Mytilus edulis	Semi-quantitative RT-PCR	Luedeking & Kohler 2002
	Mytilus galloprovincialis	Western blot	Peters et al. 1998a, b
CYP4V13 (previously CYP4C17)	*Haliotis rufescens*	Sequence	Snyder 1998
CYP4Y1	*Mytilus galloprovincialis*	Sequence	Snyder 1998
CYP10	*Lymnaea stagnalis*	Sequence	Teunissen et al. 1992
CYP11A	*Mytilus edulis*	Southern blot	Wootton et al. 1995
CYP30	*Mercenaria mercenaria*	Sequence	Brown et al. 1998
CYP320A1	*Biomphalaria glabrata*	Sequence	GenBank # AY922309
CYP322A1	*Perna canaliculus*	Sequence	K. Herron (submitted to P450 nomenclature committee in 2001)
CYP356A1	*Crassostrea gigas*	Sequence	Toledo-Silva et al. 2008

[a] The CYP family designation as defined in the above table is based on the cited author(s) assignment.
For some P450 families, caution must be taken when identifiying the presence of specific CYP families in molluscs when substantial sequence information is lacking. For example, significant genomic evidence suggests the CYP1 family is deuterostome specific (Rewitz et al. 2006; Goldstone et al. 2007).

in vertebrate systems indicate these enzymes regulate the biosynthesis and metabolism of fatty acids involved in cell signaling processes (Okita & Okita 2001 , Kikuta et al. 2002).

CYP4 genes encode several P450 enzymes capable of hydroxylating saturated and unsaturated fatty acids (e.g. palmitate, arachidonic acid and various prostaglandins) (Yamamoto et al. 1984, Matsubara et al. 1987, Sharma et al. 1989) in vertebrates. Prostaglandins are unsaturated carboxylic acids consisting of a twenty carbon skeleton, including a five membered ring, and are synthesized from the precursor arachidonic acid. Surprisingly, the highest concentrations of prostaglandins in nature have been isolated from the Caribbean gorgonians (Weinheimer & Spraggins 1969), where they function as feeding deterrents (Gerhart 1986, Pawlik & Fenical 1989, Gerhart 1991). Prostaglandin esters comprise up to 3% of the dry weight of the gorgonian *Plexaura homomalla* (Weinheimer & Spraggins 1969), with the hydroxy-methyl ester and hydroxy acid of 15(R)-prostaglandin A_2 contributing to the bulk of the chemical defense (Pawlik & Fenical 1989). *Cyphoma gibbosum* can tolerate prostaglandins produced by *Plexaura homomalla* (Gerhart 1986) and actually prefers to reside on this species of gorgonian (Chiappone et al. 2003). It seems plausible – even likely – that *Cyphoma* may use members of the CYP4 family to detoxify the prostaglandins it encounters in its diet.

While the role of the CYP4 family in fatty acid metabolism in vertebrates has been studied extensively (reviewed in Simpson 1997), additional work in terrestrial invertebrates has suggested that CYP4 isoforms may contribute to toxin metabolism. Drosophilid CYP4 isoforms have been shown to be involved in the metabolism of defensive alkaloid plant compounds (Danielson et al. 1997), illustrating that CYP4 gene functions are more diverse than mammalian functions would suggest. In contrast to most housekeeping genes, P450s involved in xenobiotic metabolism are often transcriptionally

inducible by substrates upon which they act (Whitlock 1986). Detoxification enzymes involved in allelochemical resistance should, therefore, exhibit a marked increase in expression following toxin exposure. Using a PCR-based cloning strategy, Danielson et al. (1997) was able to identify 15 novel CYPs from an isoquinoline alkaloid-resistant drosophilid. Six of these sequences displayed increased mRNA levels following exposure to isoquinoline alkaloids, while CYP4D10 was the only P450 that was strongly and specifically induced following exposure to primary host plant alkaloids but not to similar alkaloids of a rarely utilized host plant. The substrate specificity and induction response of CYP4D10 makes this detoxification enzyme the primary candidate for the observed alkaloid resistance in *Drosophila mettleri*. Other P450s that were induced to a lesser degree may still aid in allelochemical resistance through a cooperative action among multiple P450s (possibly within the same family), by allowing this generalist drosophilid to exploit a broader range of plants with chemically similar compounds. Although *C. gibbosum* is carnivorous, the association with its prey is analogous to plant-insect interactions. A generalist like *C. gibbosum* may possess an assortment of CYPs, whose expression may depend on the presence of inducers (e.g. gorgonian species-specific suite of allelochemicals) specific to each of those CYPs.

Exclusive to the molluscan genome are the CYP30 and CYP10 families (Teunissen et al. 1992, Brown et al. 1998). CYP30, originally isolated in *Mercenaria mercenaria*, is most closely related to the CYP3 family, followed by the insect CYP6, CYP9 and CYP25 families (Brown et al. 1998, Nelson 1998). While the substrate specificity of the CYP30 protein is unknown, members of the closely-related CYP3

family are involved in steroid metabolism. Steroids have consistently been isolated from many gorgonian species (Block 1974, Cimino et al. 1979, Cimino et al. 1984, Coll 1992, Rodriguez 1995, Rho et al. 2000, Tanaka et al. 2002) and may have influenced the evolution of CYPs responsible for their metabolism in molluscs. CYP10 is most closely related to mitochondrial CYP families involved in steroid synthesis (CYP24, CYP11, CYP27) (Teunissen et al. 1992). The physiological functions of CYP10 are unknown; however, it is expressed exclusively in the female gonadotropic hormone producing dorsal bodies of *Lynmaea stagnalis* (Teunissen et al. 1992).

The importance of cytochrome P450 enzymes in the metabolism of dietary plant compounds and other xenobiotics has been studied for over thirty years (Gonzalez 1989). While the primary function of P450 enzymes is to convert hydrophobic, lipid-soluble organic xenobiotics to water-soluble excretable metabolites, the phase II biotransformation enzymes such as the glutathione S-transferases also facilitate the elimination of dietary compounds from the body. Studies investigating specific activities of phase I enzymes have noted that in fish and crustaceans total P450 content is an order of magnitude greater than in molluscs. However, this trend is reversed when examining glutathione S-transferases (reviewed in Livingstone 1998). Glutathione S-transferases, in addition to P450s, have increasingly been identified in molluscs (Lee 1988, Lee et al. 1988, Fitzpatrick et al. 1995, Fitzpatrick et al. 1997, Stien et al. 1998, Kaaya et al. 1999, Vidal & Narbonne 2000, Hoarau et al. 2001, Khessiba et al. 2001, Hoarau et al. 2002, Petushok et al. 2002, Yang et al. 2002, Blanchette & Singh 2003, Le Pennec & Le Pennec 2003, Yang et al. 2004, Doyen et al. 2005, Hoarau et al. 2006, Blanchette et al.

2007, Myrnes & Nilsen 2007, Vasconcelos et al. 2007, Doyen et al. 2008) and may prove to be an equally vital adaptive response mechanism against allelochemicals in the marine environment.

Phase II – Glutathione S-transferase

Phase II biotransformation reactions involve the conjugation of a hydrophobic compound or modified metabolite from phase I with a cofactor that increases the molecular weight and polarity of the compound and facilitates excretion (Parkinson 2001). Glutathione S-transferases (GSTs) aid in this process by catalyzing the nucleophilic attack of reduced glutathione (GSH: γ-Glu-Cys-Gly) on nonpolar compounds that contain an electrophilic carbon, nitrogen, or sulphur atom, including those metabolites formed by P450 oxidation (Hayes et al. 2005). GSTs form a multi-gene family that encode cytosolic enzymes that are found in most aerobic eukaryotes and some prokaryotes and exhibit differing, but often overlapping, substrate specificities for both endogenous and exogenous compounds. Possible GST substrates include chemotherapeutic agents, insecticides, carcinogens, oxidative stress by-products and natural products (Sheehan et al. 2001, Hayes et al. 2005). Large scale genome sequencing projects have estimated the total number of GST genes for *Caenorhabditis elegans* (57), *Drosophila melanogaster* (43), and *Homo sapiens* (40) (Ding et al. 2003). To date, seven soluble cytosolic GST classes, encoding proteins of approximately 200 amino acids in length, have been identified in metazoans (e.g. Alpha, Mu, Pi, Theta, Sigma, Zeta and Omega) (Sheehan et al. 2001). Beta-class GSTs have only been

described in bacteria (Vuilleumier 1997), while the delta and epsilon classes remain insect-specific (Zhou & Syvanen 1997, Sheehan et al. 2001, Sawicki et al. 2003). GSTs within the same class share greater than 40-60% amino acid identity while those between classes typically share less than 25% identity, with the majority of the similarity at the N-terminus (Sheehan et al. 2001). In the past, various mammalian GSTs have been classified based on substrate specificity and inhibitor sensitivity; however, these techniques tend to result in overlapping values between GST classes and have proven less effective for classifying non-mammalian GSTs (Francis et al. 2001, Sheehan et al. 2001). Thus, to avoid confusion, amino acid sequences should be used for comparison between GST classes.

Each functional GST is composed of two subunits, either identical (homodimeric) or non-identical (heterodimeric) (Doyen et al. 2005). GST subunits in mammalian systems range from 199-244 amino acids in length (Hayes et al. 2005) and are also known to have two distinct binding sites: an N-terminal G site which binds glutathione, and a C-terminal H site which binds the substrate. The highly conserved N-terminal region contains a catalytically essential tyrosine, serine or cysteine residue that interacts with the thiol group of GSH. In contrast, the H site has a highly variable structure which can accommodate a wide range of substrates (Ortelli et al. 2003). The detection of alternative spliced products and the formation of heterodimers allows for the development of novel GST enzymes, which presumably increases the pool of potential substrates (Sheehan et al. 2001).

GSTs involvement in the detoxification of dietary secondary metabolites in invertebrates has been known for some time (Yu 1982, 1989, Brattsten 1992b). Many plant allelochemicals and marine natural products contain the appropriate functional groups that can be directly conjugated with GSH (Schlenk & Buhler 1988, Brattsten 1992b). Overexpression of GSTs has been suggested as a protective mechanism that invertebrates use to cope with, and adapt to, dietary allelochemicals. Various species of insects have shown 2 to 40-fold induction of GSTs upon changes in host plant diet or when exposed to plant compounds in artificial diets (Yu 1982, Brattsten et al. 1984, Yu 1984, Snyder et al. 1995). Allelochemicals such as xanthotoxin, indole derivatives, flavones, and allyl glucosinolates that induce cytochrome P450s in insects often also induce GST activity (Yu 1983, 1984, 1986, 1992). However, GSTs and P450s are not always coordinately induced. For example, plant monoterpene menthols and pinenes are known P450 inducers in the fall armyworm, yet they do not induce GST activity (Yu 1982). In contrast, in the southern armyworm coumarin is a weak P450 inducer, but strongly induces GST activity (Brattsten et al. 1984). Thus, studies intent on characterizing the responses of a single type of detoxification enzyme may miss critical information. Additionally, studies investigating GST isoforms in five different species of lepidoptera established that the degree of polyphagy was positively correlated with the number of GST isoforms found. Specialist species had only one isoform, while generalist species showed multiple forms, presumably to detoxify a broader range of metabolites (Yu 1989). In light of the variety of compounds to which *Cyphoma* can be

exposed, it will be interesting to characterize both the overall GST diversity and the differences in expression patterns among animals feeding on different gorgonian species.

Molluscan Glutathione S-transferases

GSTs have been identified with some frequency in many molluscan species (Fitzpatrick & Sheehan 1993, Fitzpatrick et al. 1995, Blanchette & Singh 1999, Vidal & Narbonne 2000, Yang et al. 2004, Doyen et al. 2005, Blanchette et al. 2007, Doyen et al. 2008). Immunoblot analysis has suggested the presence of alpha, mu and pi GST-like classes from the clam *Ruditapes decussatus* (Hoarau et al. 2002). It is now accepted that mu, alpha, and pi mammalian GST classes have a common precursor, the alpha/pi/mu class, which probably arose from theta gene duplication (Pemble & Taylor 1992). Recently, four complete cDNA sequences encoding GSTs from the pi, mu, omega, and sigma classes have been cloned from the Pacific oyster, *Crassostrea gigas* (Boutet et al. 2004). The expression of select oyster GST classes was found to be tissue-specific, as well as time- and treatment- (hydrocarbon or pesticide) dependent. Currently, many of the known molluscan GST enzymes are found within the pi class with representatives from squid, octopus, and five species of bivalves (Tomarev et al. 1991, Tomarev et al. 1993, Doyen et al. 2005); GenBank Accession # AF527010, AF227977 and EF520700).

The endogenous function of pi GSTs in molluscs has yet to be defined; however, studies in mammalian systems indicate that pi GSTs (GSTP1) are effective in the detoxification of electrophilic α, β-unsaturated carbonyl compounds produced by radical reactions, lipid peroxidation and metabolism of drugs (Berhane et al. 1994).

Additionally, prostaglandins have been found to substantially induce mammalian GST pi (GSTP1) gene transcription, mediated by a prostaglandin response element localized in the enhancer region of GSTP1 (Kawamoto et al. 2000). The cyclopentenone-type prostaglandins (e.g. PGA_2, PGJ_2) and metabolites of PGD_2, including 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 are potent inducers of GSTP1. Both the prostaglandin A and J series contain α,β-unsaturated ketones, which are very susceptible to nucleophilic addition reactions with thiols like GSH. Similar studies have confirmed the role of pi GSTs in prostaglandin conjugation (Bogaards et al. 1997). It is possible that upon ingestion of PGA_2 from its gorgonian diet, *Cyphoma gibbosum* may experience a related induction of putative prostaglandin conjugating GSTs.

While considerable attention has been focused on the role of GST in the detoxification of anthropogenic xenobiotics (Cheung et al. 2001, Hoarau et al. 2001, Blanchette & Singh 2002, Gowlan et al. 2002), there is increasing interest in molluscan detoxification of plant chemical defenses (DeBusk et al. 2000, Kuhajek & Schlenk 2003). The red alga *Odonthalia floccossa* contains a suite of brominated phenolics capable of deterring feeding in several molluscan herbivores (Kurata & Taniguchii 1997), yet it is a staple in the diet of the marine chiton, *Cryptochiton stelleri*. Biotransformation enzymes, including GSTs, were hypothesized to aid this herbivorous mollusc in utilizing *O. floccossa* as a food source despite the high concentration of brominated aromatic compounds (DeBusk et al. 2000). The elevated presence of GST in *C. stelleri* may protect the chiton against dietary metabolites while also conferring a selective advantage over other herbivores that avoid chemically defended algae.

Phase III – ABC transporters

Originally discovered for their role in chemotherapeutic drug resistance in tumor cells, the ATP Binding Cassette (ABC) transporters affect the adsorption, distribution and excretion of numerous xenobiotics. ABC transporters are a large superfamily of proteins found in bacteria to humans and are involved in the trafficking of molecules across cell membranes (Litman et al. 2001). This ATP-dependent process involves the unidirectional translocation of sugars, lipids, amino acids, peptides, metals, inorganic ions, natural products, glutathione conjugates and toxins (Croop 1998). In eukaryotes the typical structure of an ABC protein consists of two membrane-embedded transmembrane domains (TMD) and two highly conserved ATP binding domains (NBD: nucleotide binding domain) consisting of the Walker A and Walker B motifs and a signature C domain (Allikmets & Dean 1998). Sequence similarity surrounding the ATP binding site can range as high as 30% identical residues between prokaryotic and eukaryotes (Croop 1998). The TMDs contain 6 -11 membrane spanning α-helices and determine substrate specificity, while the NBDs transfer the energy released during ATP hydrolysis to the transport of the substrate across the membrane (Dean 2002). ABC transporters can be divided into seven subfamilies based on gene structure, domain order and sequence homology. Below I will describe two of these subfamilies (ABCB, ABCC) and present evidence for their involvement in xenobiotic resistance.

The first ABC transporter, permeability-glycoprotein or Pgp (a.k.a., ABCB, MDR), was identified in Chinese hamster ovary cells and has subsequently been linked to the multi-drug resistance phenotype (Juliano & Ling 1976). Since this time, P-gp-like

proteins have been described in a variety of invertebrates including sponges, mussels, oysters, clams, worms, and insects (Bard 2000). The full length P-gp gene is approximately 1280 amino acids. Each protein has two TMD consisting of six transmembrane regions and two cytoplasmic ATP binding sites (Croop 1998). P-glycoproteins are expressed in tissues instrumental in xenobiotic disposition (liver and kidney), in tissues acting as xenobiotic barriers (colon, pancreas, blood-brain barrier) and in hormone producing tissues in vertebrates (Endicott & Ling 1989). Localization of P-gp to the apical (luminal) surface of epithelial cells in various tissues support the involvement of this protein in the transport and secretion of xenobiotics (Thiebaut et al. 1987). P-glycoprotein mRNA and protein expression has been shown to increase in animals and cell cultures dosed with substrate (e.g. verapamil) (Collett et al. 2004), suggesting that this pump can act as an inducible defense and therefore aid in the protection of cells, tissues, and organisms against toxic chemicals.

Substrates for P-gp are diverse but are generally moderately hydrophobic, low molecular weight molecules with a basic nitrogen atom, cationic or neutral but never anionic. This list includes natural products, colchicines, vinca alkaloids (e.g. vinblastine, vincristine), anthracyclines (e.g. doxorubicin), verapamil, steroids (cortisol, aldosterone) and many many more (see Litman et al. 2001). Interestingly, many P-gp substrates are also substrates for cytochrome P450 and both proteins have been found to be coexpressed in tissues involved in xenobiotic absorption, such as the small intestine and liver (Watkins 1997). Several marine natural products from algae (okadaic acid, calyculin A, caulerpin), tunicates (patellamide D, lamelarins), sea hares (dolastatins), and gorgonians

(polyoxygenated steroids) have also been identified as putative substrates for P-gp (Suganuma et al. 1988, Chambers et al. 1993, Williams & Jacobs 1993, Aherne et al. 1996, Quesada et al. 1996, Schroder et al. 1998, Tanaka et al. 2002). The original role of multixenobiotic transport proteins remains unclear; however, the expression of P-gp in non-cancerous tissues and its distribution in vertebrate systems suggests a protective role against dietary secondary metabolites and environmental xenobiotics (Epel 1998).

For several years, P-gp was considered the only ABC transport protein associated with xenobiotic resistance. This all changed when a second drug-resistance-related ABC transporter, the multidrug resistance-associated protein (MRP; family ABCC), was discovered and found to share 15% amino acid identity to P-gp. In addition to the 12 transmembrane segments characterizing P-gp, the majority of MRP proteins have an additional N-terminal transmembrane domain composed of five helices and an intracellular loop making MRP roughly 1500 amino acids in length. Therefore, MRP can be thought of as having an N-terminal hydrophobic domain linked to a P-gp-like core. The tissue distribution of MRP is similar to that of P-gp, with protein expression found in the liver, kidney, gut, pancreas, bladder and tumor cell lines (Borst et al. 2000). However, unlike P-gp, the majority of MRP proteins are basolateral transporters whose operation results in the movement of compounds away from luminal surfaces into tissues or blood vessels that lie beneath the basement membrane (Evers et al. 1996).

Despite the overlap of drug resistance profiles between both transporters, the substrate selectivities of the pumps differ markedly, in that P-gp substrates are neutral or mildly positive lipophilic compounds, while MRP is able to transport neutral and anionic

lipophilic compounds and products of phase II cellular detoxification processes including glutathione conjugated compounds (Litman et al. 2001). The actual mechanism of transport, as with all ABC transporters, is not fully understood. Studies able to block intracellular GSH synthesis with BSO (buthionine sulfoximine) were able to sensitize cells to natural product anticancer compounds; however, the formation of glutathione conjugates was never detected, suggesting that conjugation may not be required to transport compounds. This led some to hypothesize a model for MRP which includes cotransport of GSH without actual GSH conjugation. Support for this model is convincing; however, it is not known to what extent MRP substrates are conjugated or cotransported (Rappa et al. 1997, Loe et al. 1998, Hipfner et al. 1999).

ABC transporter-mediated sequestration

For the most part, multixenobiotic transporters (MXT), P-gp and MRP, have been described with respect to their role in transporting compounds from the inside of the cell to the outside. However, some reports suggest that both transporters may concentrate large amounts of compounds within the cytoplasmic compartment in endocytic vesicles, effectively sequestering rather than excreting toxic compounds. The ability to sequester xenobiotics may be an alternative protective mechanism that organisms use to partition compounds away from the rest of the cell. One study investigating P-gp-mediated okadaic acid (OA) resistance in the common mussel, *Mytilus edulis*, found mussel blood cells to be highly resistant to the cytotoxic effects of OA (Svensson et al. 2003). The authors immediately suspected P-gp's involvement because of earlier reports that had

provided convincing evidence that OA was a P-gp substrate in a MDR cell line (Tohda et al. 1994, Ritz et al. 1997). However, upon measuring the accumulation in mussel blood cells of a well described P-gp substrate (vincristine) in the presence of a P-gp inhibitor (verapamil), the authors found a puzzling result. Instead of increasing the concentration of vincristine (i.e. preventing P-gp-mediated cell efflux of vincristine) as seen in gill tissue, the inhibitor actually reduced the accumulation of vincristine in mussel blood cells. Microscopy studies with the fluorescent P-gp substrate Rhodamine B confirmed the accumulation of the fluorescent dye within lysosomes and the subsequent decrease in lysosome fluorescence when incubated with verapamil. When blood cells were exposed to OA, vincristine accumulation was not significantly affected. Nevertheless, when the volume of the lysosomal compartment was measured in cells pre-exposed to OA, a significant increase was detected compared with control cells, suggesting OA sequestration within lysosomes. P-gp lysosomal sequestration of toxic pollutants has also been confirmed in F/B cells of the digestive gland in the crab *Carcinus maenas* (Kohler et al. 1998).

Sequestration of xenobiotics by ABC transporters may function as a protective mechanism against cytotoxicity; but more than this, accumulation of toxic compounds may provide protection against predation. Sequestration of dietary allelochemicals in marine invertebrates has been extensively documented (Faulkner 1992, Pennings & Paul 1993, Proksch 1994). Specialist molluscan predators, such as nudibranchs, actively take up defensive compounds from their diet (sponges or gorgonians) and use these chemicals for their own protection (Cronin et al. 1995, Avila & Paul 1997). It is not unreasonable

to hypothesize that P-gp and/or MRP may be involved in the accumulation of dietary allelochemicals in these marine predators.

Molluscan ABC transporters

For many years, molluscs (bivalves in particular) have been used in studies as indicator species to assess the health of the aquatic environment (O'Connor 2002). Research soon focused on the role of ABC transporters and their function in mediating xenobiotic resistance in marine invertebrates. Immunohistochemical studies, isolation of homologous genes, and *in vivo*/membrane vesicle activity assays all confirmed the presence of ABC transporters in molluscs (Table 2). MXT expression and activity has been detected in the gills, hepatopancreas, intestine and other tissues of aquatic invertebrates exposed to environmental chemicals (Smital et al. 2004). In addition, significant induction and subsequent return to basal levels of P-gp transport activity in bivalves exposed to pollutants in the field has been shown to occur within a 4-day period (Smital et al. 2003). This suggests that the induction of these energy-requiring defense mechanisms is tightly controlled.

Natural products of a marine origin have also been shown to modulate multixenobiotic transport activity in molluscs. A study in *Mytilus californianus* found several extracts from seaweed and phytoplankton species, mainly those of a moderately hydrophobic nature, enhanced the accumulation of a known fluorescent P-gp substrate, suggesting algal secondary metabolites may act as P-gp substrates (Eufemia et al. 2002).

Additionally, extracts from an invasive green alga species, *Caulerpa taxifolia,* caused the accumulation of fluorescent MXT substrates in the gills of exposed bivalves and

Table 2. Biochemical and molecular evidence for molluscan ABC transporters

ABC subfamily designation	Species	Evidence	Reference
ABCB subfamily or P-glycoprotein (Pgp)	*Anodonta cygnea*	Western blot, Fluorescent dye assay	Smital et al. 2000
	Brachidontes pharaonis	Sequence	GenBank # AJ972911, Feldstein et al. 2006
	Corbicula fluminea	Western blot, ^{3}H-vincristine accumulation assay	Waldmann et al. 1995, Achard et al. 2004
	Crassostrea gigas	Sequence, Western blot, Southern blot	GenBank # AJ422120; EU073425, Minier et al. 1993
	Crassostrea virginica	Fluorescent dye assay, Western blot, Sequence	GenBank # AY319412, Keppler & Ringwood 2001
	Dreissena polymorpha	Sequence, Fluorescent dye assay	GenBank # AJ506742, Smital et al. 2000, Smital et al. 2003
	Monodonta turbinata	Western blot, Fluorescent dye assay	Smital et al. 2000
	Mytilus californianus	Western blot, Fluorescent dye assay, Sequence	Cronwall et al. 1995, Eufemia & Epel 2000, Luckenbach & Epel 2008
	Mytilus edulis	Western blot, Sequence, Fluorescent dye assay	GenBank # AF159717, Minier et al. 1993, Minier & Moore 1996, Luedeking & Kohler 2002, Svensson et al. 2003
	Mytilus galloprovincialis	Western blot, Fluorescent dye assay, ^{3}H-vincristine accumulation assay, Sequence	Kurelec 1995, Galgani et al. 1996, Smital et al. 2000, Smital et al. 2003, Franzellitti & Fabbri 2006
	Patella lusitanica	Fluorescent dye assay	Smital et al. 2000
	Perna perna	Fluorescent dye assay, Western blot, Sequence	GenBank # AF150878, Franco et al. 2006
	Saccostrea forskali	Western blot, Fluorescent dye assay, Sequence	Kingtong et al. 2007
	Viviparus viviparus	Fluorescent dye assay	Smital et al. 2000
	Unio pictorum	Sequence	GenBank # AY857552
ABCC subfamily or Multidrug resistance-associated protein (MRP)	*Crassostrea gigas*	Sequence	GenBank # AJ438990
	Mytilus californianus	Fluorescent dye assay, Sequence	Luckenbach & Epel 2008
	Mytilus edulis	Sequence, Northern blot	GenBank # AF397142-3, Luedeking et al. 2005
	Mytilus galloprovincialis	Sequence	Franzellitti & Fabbri 2006
	Saccostrea forskali	Western blot, Fluorescent dye assay, Sequence	Kingtong et al. 2007

subsequent inhibition of multixenobiotic resistance (Smital et al. 2004). Evidence also suggests that glutathione-conjugated natural products may be transported by molluscan MRPs. Human MRP1 is known to transport the glutathione conjugate of prostaglandin A_2 (GS-PGA_2) (Evers et al. 1997, Ishikawa et al. 1998). In gorgonians, PGA_2 and derivatives are potent feeding deterrents and have been isolated in high concentrations (Gerhart 1984). Upon their ingestion these compounds may inhibit the inherent multixenobiotic transporter defenses of potential consumers and make them more susceptible to these and other gorgonian chemical defenses. Interestingly, molluscan

predators like *C. gibbosum*, which feed exclusively on gorgonians, do not seem affected by gorgonian chemistry and may have evolved sophisticated MXT to avoid cytotoxicity and exploit an abundant food source untouched by other reef predators.

Rationale

Understanding how organisms deal with potentially toxic or fitness-reducing allelochemicals has significance for understanding patterns of predation and herbivory in the marine environment. Cytochrome P450 and glutathione *S*-transferase enzymatic activity has been extensively documented in many marine invertebrates (Snyder 2000, Le Pennec & Le Pennec 2003, Rewitz et al. 2006); however, as yet, no genes specifically involved in the detoxification of dietary allelochemicals have been sequenced. Similarly, little is known about dietary allelochemicals that act as substrates for P-glycoprotein or multidrug resistance protein in marine invertebrates. The focus of this research was to characterize the diversity and dietary regulation of cytochrome P450s, glutathione *S*-transferases, and multixenobiotic transporters in two marine molluscs and to identify dietary natural products that act as substrates for these proteins.

Chapter 2 describes a proteomic approach used to identify the diversity and phylogenetic relatedness of GSTs in *Cyphoma*. Chapter 3 investigates the diversity of putative CYP allelochemical metabolizing genes, their transcriptional responsiveness to host-gorgonian allelochemicals and their enzymatic activity toward allelochemical analogs. Chapter 4 explores the biochemical interactions among gorgonian extracts/pure compounds with *Cyphoma* GSTs, further relating physiological enzyme activity with

ecologically relevant concentrations of gorgonian natural products. Finally, Chapter 5 examines the distribution and activity of two diverse subfamilies of multixenobiotic resistance proteins in both generalist and specialist molluscs to assess the potential importance of these efflux proteins in mediating allelochemical transport.

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Chapter II

Proteomic identification, cDNA cloning, and enzymatic activity of glutathione *S*-transferases from the generalist marine gastropod, *Cyphoma gibbosum*

Abstract

Glutathione S-transferases (GST) are key detoxification enzymes involved in the biotransformation and excretion of reactive electrophilic compounds including natural products. GSTs were characterized from the digestive gland of the tropical gastropod, *Cyphoma gibbosum*, to investigate the possible role of these enzymes in conferring resistance to allelochemicals present in this consumer's gorgonian coral diet. I identified the collection of expressed cytosolic GST classes in *C. gibbosum* using a proteomic approach involving affinity chromatography, high performance liquid chromatography, electrophoresis, nanospray liquid chromatography-tandem mass spectrometry (LC-MS/MS) and MS/MS sequencing. Two major GST subunits, constitutively expressed in digestive gland tissues, were identified as putative mu-class GSTs most closely related to GSTs from abalone and oyster. One minor GST subunit was identified as a putative theta-class GST, apparently the first theta-class GST identified from a molluscan source. Two mu-class GST cDNAs, CgGSTM1 and CgGSTM2, were isolated by RT-PCR using primers derived from peptide sequences. The full-length cDNA sequences encode proteins of 215 amino acids in length that share 78% amino acid identity. Phylogenetic analysis establishes these as mu-class GSTs and reveals a mollusc-specific subclass of the GST-mu clade. Homology of *C. gibbosum* digestive gland GSTs to mammalian and bacterial isoforms suggests a possible role in the biotransformation of gorgonian natural products such as organohalogens and prostaglandins. These results provide new insights into metazoan GST diversity and the biochemical mechanisms used by marine animals to cope with their chemically defended prey.

Introduction

Glutathione S-transferases (GSTs, EC 2.5.1.18) form a large superfamily of multifunctional enzymes capable of conjugating a broad range of toxic electrophilic xenobiotics with glutathione (Sheehan et al. 2001). GSTs function primarily as detoxification enzymes, generally rendering the resultant products more water soluble (nonreactive conjugate), thereby facilitating excretion. The catalytic versatility and diversity of GSTs can be attributed to the nonspecific nature of the hydrophobic substrate binding site (H-site) and the extensive gene duplication and divergence that have occurred in this superfamily (Ivarsson et al. 2003, Pearson 2005). The soluble GSTs in metazoans are divided into seven classes (alpha, mu, pi, theta, sigma, zeta, and omega) based on sequence identity, immunological and kinetic properties (Sheehan et al. 2001, Nebert & Vasiliou 2004, Hayes et al. 2005). While the majority of GST enzymatic characterization has focused on mammalian forms, non-vertebrate models offer an exciting opportunity to examine the evolution of GSTs and their adaptive responses to environmental chemicals, including natural products.

Biochemical adaptations involving xenobiotic response genes, such as GSTs, may help explain consumer resistance to dietary chemical threats, providing insight into enzymatic mechanisms underlying consumer foraging patterns. For example, the ability of terrestrial invertebrates (insects) to tolerate naturally occurring dietary toxins (i.e. allelochemicals in their host plants) has been linked in part to high constitutive activity of GSTs (reviewed in Li et al. 2007). The induction of GSTs in response to dietary host allelochemicals may serve as an additional adaptive mechanism to protect against toxicity. While the role of GSTs in plant-herbivore interactions has clearly influenced the

ecology of terrestrial consumers, far less is known about the detoxification enzymes that allow marine invertebrates to exploit allelochemically defended prey.

Only a few studies have addressed the induction of GSTs upon exposure to allelochemicals in marine invertebrates (Vrolijk & Targett 1992, DeBusk et al. 2000, Kuhajek & Schlenk 2003). One such study (Vrolijk & Targett 1992) examined GST activity in the digestive gland of a generalist gastropod, *Cyphoma gibbosum*, which exclusively feeds on several families of chemically defended gorgonian corals. The authors reported significantly higher GST activity in field-collected *C. gibbosum* feeding on gorgonians *Gorgonia ventalina* and *Briareum asbestinum,* suggesting that GST expression varies in response to different suites of gorgonian allelochemicals. Additionally, GST activity from *C. gibbosum* cytosolic preparations was among the highest ever reported from a molluscan digestive gland and was similar to values described from *Papilio polyxenes*, a specialist insect that feeds solely on chemically defended plants (Lee 1991, Lee & Berenbaum 1992). In a subsequent study (Cronin et al. 1995), thin-layer chromatographic profiles of nonpolar tissue extracts from *C. gibbosum* feeding on *G. ventalina* did not mirror those of its octocoral prey, lending further support to the idea that this gastropod predator has the capacity to biotransform dietary compounds to readily excretable metabolites. However, the GST isoforms responsible for the detoxification of gorgonian allelochemicals are not known; and in general GST diversity in molluscs - including gastropods - is poorly understood.

GST cDNA sequences representing alpha, mu, pi, omega, and sigma GST classes have been identified in molluscs. The majority of sequences are grouped within the pi class, with representatives from cephalopods (squid, octopus), and seven bivalve species

(Tomarev et al. 1991, Tomarev et al. 1993, Boutet et al. 2004, Yang et al. 2004, Doyen et al. 2005, Hoarau et al. 2006, Munasinghe et al. 2006, Myrnes & Nilsen 2007, GenBank® Accession No. EF194203, EF520700, DQ530213, DQ530212). The endogenous function of pi-class GSTs in molluscs has yet to be defined; however, studies in mammalian systems indicate that pi GSTs are more participatory than other GST classes in the detoxification of prostaglandins and other electrophilic α, β-unsaturated carbonyl compounds (Berhane et al. 1994, Bogaards et al. 1997). Prostaglandins are potent signaling molecules involved in the production of pain and fever (Malan & Porreca 2005), the regulation of blood pressure/coagulation (Egan & FitzGerald 2006) and reproduction (Goldberg & Ramwell 1975). The highest concentrations of prostaglandins in nature have been found in Caribbean gorgonians (Weinheimer & Spraggins 1969), where the acetoxy acids, hydroxyl methyl esters and hydroxyl acids of prostaglandin A_2 function as feeding deterrents against generalist reef fish (Gerhart 1984, Pawlik & Fenical 1989). Prostaglandins in the A series especially can significantly induce GST activity in mammalian cells (Uchida 2000). I hypothesize that *C. gibbosum* GSTs may conjugate gorgonian allelochemicals, like prostaglandins, potentially alleviating their toxicity.

The objective of the present study was to isolate and characterize the GSTs in *C. gibbosum* digestive gland that may protect this marine consumer from allelochemicals found in its prey. Initially, I targeted pi-class GSTs because of their role in prostaglandin metabolism. However, attempts to obtain pi-class GSTs from *C. gibbosum* using RT-PCR with degenerate primers designed from an alignment of molluscan GST pi cDNA sequences (Doyen et al. 2005) failed to yield any GST sequences. Therefore, I initiated a

proteomic approach involving affinity chromatography coupled with HPLC and mass spectrometry to provide an unbiased assessment of GST protein diversity in the digestive gland of *C. gibbosum*. Here I report the identification of several mu-class GST proteins in *C. gibbosum* digestive gland, the cloning and phylogenetic characterization of two complete mu-class GST cDNAs and partial peptide sequences of a theta-class GST, apparently the first member of this class identified from a mollusc. These results contribute to a better understanding of GST diversity in molluscs and of the biochemical resistance mechanisms used by marine consumers to cope with chemical defenses in their prey, providing insight into patterns of predation and herbivory in the marine environment.

Materials and Methods

Reagents

CDNB, dithiothreitol (DTT), potassium phosphate, potassium chloride, EDTA, protease inhibitor cocktail (4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin, bestatin hydrochloride, E-64, leupeptin, pepstatin A), SDS, boric acid, NaCl, sodium acetate, GSH, GSH-agarose were purchased from Sigma (St. Louis, MO). Bradford reagents and molecular weight standards for protein gels were purchased from BioRad (Hercules, CA). Novex® Tris-glycine gels and Sample Treatment Buffer were purchased from Invitrogen (Carlsbad, CA). Silver SNAP® Stain for Mass Spectrometry was purchased from Pierce (Rockford, IL).

Animals

Adult *Cyphoma gibbosum* (ca 2-3 cm length) were collected from five shallow reefs (< 20m) near the Perry Institute of Marine Science (PIMS), Lee Stocking Island, Exuma Cays, Bahamas in January 2006 and transported to wet laboratory facilities provided by PIMS. The feeding assays are described in detail in Chapter 3. Individuals were allowed to feed on a control diet (e.g. alginic acid and freeze-dried squid paste (O'Neal & Pawlik 2002) or one of six gorgonian diets (*Briareum asbestinum, Eunicea mammosa, Gorgonia ventalina, Pseudopterogorgia acerosa, Pseudopterogorgia americana, Plexaura homomalla*) for four days. The length (4 days) of the feeding assay was chosen based upon two previous studies that measured *C. gibbosum* mean residence time on gorgonian colonies (Harvell & Suchanek 1987, Ruesink & Harvell 1990) and the induction/decay kinetics of GST expression (Pickett et al. 1982). Upon completion of the feeding assay the digestive glands were immediately dissected and either preserved in RNALater® (Ambion, Austin, TX) or frozen in liquid nitrogen and transported back to the Woods Hole Oceanographic Institution. Tissues were maintained at -80°C until further analysis.

Enzyme purification

Individual digestive glands (n = 42) were homogenized 1:4 (w/v) in ice-cold homogenization buffer (0.1 M potassium phosphate, 1 mM EDTA, 1 mM DTT, 1.15% potassium chloride, protease inhibitor cocktail (1X); pH 7.5) with an IKA Ultra Turrax T8 homogenizer (Wilmington, NC) for 30 sec on ice. All subsequent steps were carried out at 4°C. Cytosol was isolated by centrifugation of the crude homogenate at 750 x *g*

for 10 min then, without stopping, at 12,000 x *g* for 10 min using a Beckman J2-21 centrifuge (Fullerton, CA). The supernatant was carefully removed, avoiding both the fatty layer and the pellet and transferred to an ultracentrifuge tube and centrifuged at 100,000 x *g* for 70 min using a Beckman L8-60M ultracentrifuge (Fullerton, CA). An aliquot of the supernatant was taken for cytosolic protein determination using the BCA protein assay method (Smith et al. 1985) with BSA as the standard and the remaining cytosol was flash frozen in liquid nitrogen until further analysis. Samples were stored for several months in this manner suffered no apparent loss of enzymatic activity.

GSTs were purified using size exclusion chromatography, affinity chromatography and HPLC following a modified method from (Donham et al. 2005) . Cytosolic samples from individual snail digestive glands were purified separately. Initially, a subset of cytosol samples (n = 8) were used to determine the optimal buffer conditions and volumes for GST purification. Eluted 1-mL fractions from both size-exclusion and affinity columns were sampled for activity towards CDNB and assayed for protein using the Bradford assay (Bradford 1976). These results helped streamline the GST purification process for the remaining cytosol samples.

A PD-10 desalting column (bed volume 8.3 mL, bed height 5 cm, 5K NMWL) (GE Healthcare, Piscataway, NJ) containing Sephadex G-25 matrix was equilibrated in Buffer A (50 mM Tris buffer, 1 mM EDTA, 1 mM DTT; pH 6.0) and 2.5 mL of crude cytosol were applied to the column. Cytosolic samples less than 2.5 mL were brought up to this volume with Buffer A and then applied to the column. GST proteins were eluted by gravity with Buffer A and fractions containing GST activity were combined (approx. 6 mL of elute) and then applied to a GSH-agarose affinity column (bed volume 0.5 mL, 0.8

x 4 cm i.d.) equilibrated in Buffer A. The affinity column was washed with 7 mL of Buffer B (Buffer A + 0.5 M NaCl) to rinse away non-specific proteins. Retained GSTs were then eluted with 5 mL of Buffer C (50 mM Tris-base, 1 mM EDTA, 1 mM DTT, 0.5 M NaCl, 50 mM glutathione; pH 9.5) and fractions containing GST activity were then combined, buffer exchanged to low salt concentration, and concentrated with Amicon Ultra-4 centrifugational filters (5K NMWL membrane; Millipore, Billerica, MA). Protein concentrations of Amicon concentrates were determined with the NanoOrange protein quantitation kit (Molecular Probes, Eugene, OR).

Affinity-purified GSTs from individuals feeding on the same diet were pooled (100 µL injection volume) and injected onto a reverse phase Vydac protein/peptide column (model #218 TP 52; C18 µm 250 mm x 2.1 mm) and separated on a Waters 600 MultiSolvent Delivery System, with a flow rate of 0.5 mL/min. Peaks were detected using a Waters 2487 Dual Wavelength Absorbance Detector (λ = 214 nm). Mobile phase A consisted of 38% acetonitrile, 62% water and 0.1% trifluoroacetic acid (TFA). Mobile phase B consisted of 80% acetonitrile, 20% water and 0.1% TFA. The initial mobile phase consisted of 100% A. GST subunits were separated using a linear gradient from 0 to 40% B in 22 min, and 40 to 100% B in 37 min. Peaks were hand-collected, centrifugally evaporated to dryness, resuspended in 1X sample treatment buffer and separated by 12% Novex® Tris-glycine SDS-PAGE gel electrophoresis according to manufacturer's instructions (Invitrogen, Carlsbad, CA). Bands were visualized using the SilverSNAP® staining kit for mass spectrometry, excised from the gel and delivered to the UC-Davis Proteomics Facility, Davis, CA for in-gel digestion and LC-MS/MS analysis.

Protein in-gel digestion

Protein bands were reduced and alkylated according to (Shevchenko et al. 1996). Proteins were reduced with 10 mM DTT in 100 mM NH_4HCO_3 (pH 8, 55°C) for 1 h, then alkylated with 55 mM iodoacetamide in 100mM NH_4HCO_3 for 45 min in the dark at room temperature. Excess reagent was removed, and gel pieces were washed with 100 mM NH_4HCO_3 and partially dehydrated with acetonitrile; complete dehydration was then performed *in vacuo*. Finally, proteins were digested in 50% NH_4HCO_3 containing sequence grade trypsin (Promega, Madison, WI) at a final concentration range of 10-25 ng μL^{-1} (37°C) for 17 h. Peptides were extracted once with 0.1% TFA in water and once with 5% formic acid:acetonitrile (1:1). The extraction volume was carefully controlled to never exceed 50 µL for mass spectrometric analysis.

Nanospray LC-MS/MS and database analysis

The trypsin-digested samples were analyzed using a Eksigent Nano LC 2-D system (Eksigent, Dublin, CA) coupled to an LTQ ion trap mass spectrometer (Thermo-Fisher, San Jose, CA) interfaced with a New Objective Picoview Nano-spray ionization source (Woburn, MA) to identify peptide fragments. Digested peptides were loaded on a reverse-phase Agilent Nano-trap (Zorbax 300SB-C18, 300Å, Agilent Technologies, Santa Clara, CA) at a loading flow rate of 5 µL/min for 10 min. The buffers used for the reverse-phase chromatography were 0.1% formic acid in water (solvent A) and 95% acetonitrile in 0.1% formic acid in water (solvent B). Peptides were eluted from the trap and chromatographically separated on a reverse-phase capillary column (Pico Frit, 75 µm x 15 cm, tip 5 µm: New Objective) packed in-house with Magic C18 AQ (3 µm,

100Å:Michrom BioResources, Auburn, CA) with a 40-min linear gradient of 2-80% solvent B (as described below) at a flow rate of 300nL min^{-1}. The LTQ parameters were as follows: electrospray potential, 1.8 kV; source temperature, 180°; collision energy, 35%; dynamic exclusion duration, 1min. The MS survey scan followed by ten MS/MS scans were consecutively acquired over the LC gradient. Upon completion of an LC-MS/MS run, the MS/MS spectra from each survey were charge state deconvoluted and searched against the non-redundant NCBI protein database using BioWorks version 3.3 (Thermo Scientific, Waltham, MA) and MASCOT (Matrix Science, London, UK) and against the MSDB database (Imperial College, London, UK) using GPM software (http://www.thegpm.org). Protein fragments with significant hits to eukaryotic GSTs were then manually validated.

GST activity assay

Enzyme activity was measured using CDNB as a substrate by the method of (Habig et al. 1974) optimized for *C. gibbosum* (Vrolijk & Targett 1992) in a microplate format. The reaction mixture (in a final volume of 200 µL) contained 0.1 M potassium phosphate buffer, 1.0 mM EDTA, pH 7.5, 1 mM CDNB, 1 mM reduced glutathione (GSH) and 2µg of protein. CDNB was solubilized in ethanol and constituted 1% of the final reaction mixture volume. The reaction incubated at 25°C was initiated by the addition of CDNB and performed in triplicate. The conjugation of CDNB with GSH was measured as the increase in absorbance at 340 nm ($\Delta\varepsilon_{340}$ 0.00503 μM^{-1} cm^{-1}) using a tunable microplate reader (Versamax, Molecular Devices, Sunnyvale, CA). Activity was

calculated using protein concentrations determined via the Bradford assay with BSA as a standard.

RNA isolation and cDNA synthesis

Total RNA was purified from *C. gibbosum* digestive glands using the RNeasy Maxi Kit and DNAse treated using a RNase-free DNAse Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The integrity of each RNA sample was checked by electrophoresis on 1% agarose-formaldehyde gel in MOPS buffer and visualized with ethidium bromide under UV light. Total RNA concentration was determined using a Nanodrop spectrophotometer (Wilmington, DE). Poly(A)+ RNA was isolated using the MicroPoly(A)Purist mRNA purification kit (Ambion, Austin, TX) according to the manufacturer's instructions. Poly(A)+ RNA was pooled from seven individuals feeding on either a control diet or one of six gorgonian species (0.14 μg poly(A) RNA/individual) to ensure representation of all GSTs expressed under various dietary conditions. One microgram of pooled RNA was primed with modified oligo (dT) primers and used to create an adaptor-ligated ds cDNA library synthesized using the Marathon cDNA Amplification Kit (BD Biosciences, Palo Alto, CA) according to the manufacturer's instructions.

Primer design, RACE-PCR, cloning, sequencing

Degenerate primers were designed to *C. gibbosum* GST peptide sequences identified from LC-MS/MS with homology to mu-class GSTs from abalone (*Haliotis discus discus*) and oyster (*Crassostrea gigas*) (GSTm_R10) (Table 1), and theta-class

GSTs from sea bream (*Pagrus major, Sparus aurata*), plaice (*Pleuronectes platessa*) and bass (*Micropterus salmoides*) (primers not shown). Primers were obtained from Sigma Genosys (St. Louis, MO). PCR products were generated by rapid amplification of cDNA ends (RACE) using GST degenerate primers in combination with specific oligonucleotides designed to adaptor sequences located on the 3' and 5' ends of the cDNA. Amplification of PCR products was carried out according to the Advantage 2 PCR Enzyme Kit (Clontech, Mountain View, CA) and cycling parameters were as follows: 94°C for 30 sec; 30 cycles of 94°C for 5 sec, annealing at 55°C for 30 sec, extension at 68°C for 2 min. Primers were used at the following concentrations: 10 μM of adaptor primer and 100 μM of degenerate primer in a 50 μL reaction. Sequences obtained by RACE were then used to design specific primers (GSTm_F13 and GSTm_F15) to clone full-length *C. gibbosum* GST cDNAs. PCR products were cloned into pGEM-T (Promega, Madison, WI) and sequenced in both directions using an ABI 3730XL capillary sequencer at the Keck facility of the Josephine Bay Paul Center for Comparative Molecular Biology & Evolution at the Marine Biological Laboratory (Woods Hole, MA). A minimum of twelve clones were sequenced for each PCR fragment.

Sequence analysis

Clones were grouped based on similar nucleotide sequence using Sequencher (Gene Codes Co., Ann Arbor, MI) and used to generate a consensus sequence that was compared to known GST coding sequences using BLASTX. Multiple alignments of deduced amino acid sequences were performed using ClustalW (Thompson et al. 1994).

Ambiguous alignment positions were excluded from further analyses. Phylogenetic relationships were investigated using Bayesian techniques as implemented in the computer program MrBayes (v 3.1.2; Ronquist & Huelsenbeck 2003). MrBayes estimates posterior probabilities using Metropolis-Hastings coupled Monte Carlo Markov chains (MC^3). MC^3 estimates were performed with uninformative prior probabilities using the WAG model of amino acid substitution (Whelan & Goldman 2001) and prior uniform gamma distributions approximated with four categories (WAG+I+Γ). Four incrementally heated, randomly seeded Markov chains were run for $3x10^6$ generations, and topologies were sampled every 100^{th} generation. The MC^3 burnin values were conservatively set at $1x10^6$ generations. Posterior probabilities of topologies and clades were estimated from the sampled topologies after removal of the initial MC^3 burnin.

Results

GST purification and activity

Cytosol was isolated from the digestive glands of individual *C. gibbosum* feeding for four days on one of six gorgonian diets (n = 4-7 snails per gorgonian diet) or a control diet (n = 13 snails), resulting in a mean total protein yield of 125.6 ± 52.8 mg g^{-1} digestive gland wet weight. *C. gibbosum* digestive glands collected for this study averaged 0.22 ± 0.11 g $snail^{-1}$ (n = 42; mean ± SD). Glutathione transferase activity of crude cytosol ranged from 0.57 to 5.82 U mg^{-1} protein, with an average of 2.67 ± 1.27 U mg $protein^{-1}$ (n = 42), using CDNB as a substrate. Following size exclusion chromatography and affinity purification of the GSTs from *C. gibbosum* digestive gland, an average of 25 ± 14 μg of GST protein was collected from each digestive gland (n =

36), representing approximately 0.2% of the total cytosolic protein. A 220-fold purification of GST protein was obtained using size-exclusion and affinity chromatography (Table 2). Representative GST elution profiles from the Sephadex G-25 column and the GSH-agarose columns are presented in Figure 1.

Gel electrophoresis and GST identification

To provide an initial characterization of affinity-purified proteins, bound protein fractions obtained from GSH-agarose affinity columns from two individuals (feeding either on *P. americana* or the control diet) were concentrated, combined, and 15 µL of the pooled sample was analyzed on a 12% Novex® Tris-glycine SDS-PAGE gel (Figure 2). Three distinct bands ranging in size from 21 to 24 kDa were detected by silver staining and excised from the gel for identification via LC-MS/MS. All three bands contained peptide sequences that closely matched those of mu-class GSTs in the database (Table 3). This pooled GST affinity-purified fraction was used as standard for subsequent SDS-PAGE gels.

HPLC and LC-MS/MS based GST identification

Affinity-purified fractions from individual digestive glands were concentrated and then equal volumes of affinity-purified GSTs were pooled within each snail diet for further HPLC separation of GST subunits. HPLC analysis of affinity-purified GSTs pooled within snail diet identified a total of fourteen unique (2 major and 12 minor) peaks. The relationship between snail diet and GST subunit expression will be described elsewhere (Chapter 4). Here, I present the proteomic characterization of HPLC separated

GST subunits. Figure 3 shows a representative HPLC separation of digestive gland GST subunits from four *C. gibbosum* feeding on *B. asbestinum*. For peak collection purposes, the fourteen peaks were grouped into seven fractions (B-H; A= injection peak) (Figure 3).

The HPLC fractions were separated on 12% Tris-glycine SDS-PAGE gels (Figure 4) and bands were excised for proteomic analysis. Peptide sequences were determined for fractions B-H using nanospray LC-MS/MS and identified by comparison to proteins in the Genbank® database. Fractions D and F, with elution times of 18 and 22 minutes, respectively, represent the two major GST subunits. Both of these fractions yielded peptides that closely matched mu-class GSTs (Table 3; Figure 5). Peptides in the remaining fractions also matched GST mu-class proteins, with the exception of fraction B, which yielded eight peptides that matched theta-class GSTs from fish (Table 3; Figure 6). Manual validation of LC-MS/MS spectra verified five of the *C. gibbosum* peptide sequences as identical to the fish GST-theta peptides (Table 3).

C. gibbosum cDNA cloning and sequence analysis

To obtain full-length cDNA sequences for *C. gibbosum* GSTs, polypeptides identified by mass spectrometry were used to design degenerate primers. The mu-class GST degenerate primer (GSTm_R10), designed to the peptide sequence AYMASDK, yielded two 600-bp fragments that were confirmed by BLAST searches to be partial cDNAs encoding mu-class GSTs. Complete cDNA sequences were generated by 3' RACE, revealing open reading frames encoding predicted proteins of 215 amino acids each. The predicted proteins displayed 61% and 62% amino acid identity to Pacific oyster GST mu for transcript 1 (CgGSTM1; GenBank® Accession No. EU008563) and

transcript 2 (CgGSTM2; GenBank® Accession No. EU008562), respectively. The two *C. gibbosum* GST mu cDNAs are 78% identical and 88% similar (BLOSUM62) to each other at the protein level. Translated nucleotide sequences predict a molecular mass of 25.0 kDa for CgGSTM1 and 25.2 kDa for CgGSTM2. Figure 5 shows an alignment of these two sequences with several invertebrate and vertebrate mu-class GST sequences. Amino acid residues involved in glutathione binding as determined using the Pfam database (Marchler-Bauer et al. 2007) are entirely conserved among *C. gibbosum*, vertebrate and invertebrate sequences included in the alignment, while substrate binding sites are poorly conserved.

Two *C. gibbosum* tryptic peptides identified in the LC-MS/MS analysis (ITQSN AILR and AYMASDK), were found to be identical to corresponding *C. gibbosum* GST translated nucleotide sequences (nucleotides 583-603 in CgGSTM1 and CgGSTM2; and 205-231 in CgGSTM1, respectively) (Table 3).

To infer relationships between *C. gibbosum* predicted protein sequences and other GST sequences, a phylogenetic tree was constructed using Bayesian techniques. *Cyphoma* GSTM1 and GSTM2 clearly group within the mu-class GST clade and appear most closely related to other molluscan mu GSTs, which together form a strongly supported subgroup within this clade (Figure 7).

Discussion

Dietary toxins present physiological challenges to marine consumers, such as *Cyphoma gibbosum*, that feed solely on chemically defended gorgonian prey. The high concentration of allelochemicals in gorgonian corals (Paul 1992, O'Neal & Pawlik 2002),

coupled with the findings that digestive glands of *C. gibbosum* – which feed exclusively on gorgonians - contain high levels of GST activity (Vrolijk & Targett 1992), prompted an investigation to identify and biochemically characterize those GST enzymes possibly responsible for detoxifying gorgonian allelochemicals.

The levels of cytosolic GST activities in *C. gibbosum* digestive gland (Table 2 and Vrolijk & Targett 1992) are among the highest recorded for any molluscan tissue (reviewed in Vrolijk & Targett 1992, Le Pennec & Le Pennec 2003). The majority of studies reporting GST activity from molluscs have used GST activity measurements as an indicator of pollutant exposure (Sheehan et al. 1995, Fitzpatrick et al. 1997, Kaaya et al. 1999, Hoarau et al. 2006, Verlecar et al. 2006). Although the level of GST activity in *C. gibbosum* is high even in comparison to that of molluscs exposed to pollutants known to induce GST expression (Boryslawskyj et al. 1988, Lee 1988, Fitzpatrick et al. 1995), this high activity is unlikely to be a response to anthropogenic contamination, given the remote site of collection. Rather, the high GST activity may reflect an adaptation that facilitates consumption of allelochemical-rich prey. Whether high GST activity is common among marine species that feed exclusively on chemically defended food is unknown, and may depend upon the specific suite of allelochemicals present in the diet. Regardless of its origin, the high GST activity in *C. gibbosum* suggested that this species would be a rich source of GST enzyme(s), enabling proteomic studies to further characterize GSTs potentially involved in allelochemical metabolism.

The results of the proteomic and molecular analyses in the present study clearly show that the predominant cytosolic GSTs expressed in *C. gibbosum* digestive gland are mu-class GSTs. These include the major HPLC peaks (fractions D and F) as well as

several minor peaks. According to the current system of GST classification, GSTs sharing greater than 60% identity fall within the same class, while those with less than 30% identity are assigned to separate classes (Sheehan et al. 2001). The classification of *C. gibbosum* GSTs as members of the mu class is supported by both the homology searches of *C. gibbosum* tryptic peptides (Table 3) and the phylogenetic analysis of translated *C. gibbosum* GST cDNAs (Figure 7). The presence of mu-class GSTs in molluscs had been predicted based on immunoblot analysis (Sheehan et al. 1995, Vidal et al. 2002, Hoarau et al. 2004), but only recently have molluscan GST mu nucleotide sequences been determined (Boutet et al. 2004, Myrnes & Nilsen 2007; Genbank® Accession No. ABF67506). Our identification of two GST cDNAs in *C. gibbosum* provide the first published description of GST-mu forms from gastropods. Phylogenetic analysis reveals a mollusc-specific subclass within the GST-mu clade.

Proteomic results indicate the possibility of additional GST mu subunits beyond the two cDNAs identified here. Only two of the twelve *Cyphoma* tryptic peptides identified as matching abalone and oyster mu-class GSTs were found to be encoded by the two cDNA clones isolated from *C. gibbosum*. Not surprisingly these peptides corresponded to fairly conserved regions of the GST protein. However, three peptides (KAAYFEALPAK; SFLGDQQFFAGSK; and IMQPGSLDAFPTLLAFMGRIEALPAIK) identified as matching abalone GSTM are quite divergent from the translated *C. gibbosum* sequences, indicating that at least one, and possibly as many as three, additional GST mu subunits are present in *Cyphoma*. HPLC analysis of affinity-purified extracts suggests that there may be additional mu-class subunits, perhaps as many as thirteen (represented by the 13 peaks in fractions C-H,

Figure 3), the majority of which are minor components. In addition, visualization of hand-collected HPLC fractions by SDS-PAGE and silver staining (Figure 4) revealed the major peak in fraction F to be a composite of two separate GST subunits that were unresolvable by HPLC. Peak F from affinity-purified extracts from each of the seven diets gave this similar double banding pattern (data not shown), indicating that both subunits are universally expressed regardless of snail diet. Both bands were later confirmed to be mu-class GSTs by LC-MS/MS, thus increasing the count of potential mu-class subunits identified by HPLC analysis to fourteen. Whether the HPLC peaks represent truly unique subunits or HPLC-resolved post-translationally modified variants of the same subunit remains unclear. Multiple GST mu subunits in a single species are not uncommon; five distinct mu-class subunits have been identified from humans (Sheehan et al. 2001). Overall, our results provide conclusive evidence for at least three distinct GST-mu isoforms, and perhaps more, in *C. gibbosum*. Interestingly, mu-class GST subunits are capable of forming heterodimers (Hayes & Pulford 1995). Whether *Cyphoma* GST subunits form heterodimers is unknown, but the formation of heterodimeric GSTs has been suggested as a way of increasing the range of substrates that might be acted on by a limited set of GST isoforms (Sheehan et al. 2001).

In this study, I identified a theta-class GST from *C. gibbosum* digestive gland and by manual validation confirmed the sequence of five peptides to be identical to theta and theta-class-related GSTs from fish. The HPLC fraction B shows a single peak, suggesting that only one subunit is expressed. Several attempts to amplify theta-like GSTs from *C. gibbosum* digestive gland mRNA with degenerate primers designed to these peptide sequences were not successful.

The identification of putative a theta-class GST in *C. gibbosum* was unexpected. To the best of our knowledge, no theta-like GSTs have been identified previously in a molluscan species, although theta-class GSTs exist in vertebrates, arthropods, polychaetes, algae and bacteria (Pemble & Taylor 1992, Rhee et al. 2007). Theta-class GSTs in general have been notoriously hard to identify because they normally do not bind to affinity matrices such as GSH-agarose (Sheehan et al. 2001). In addition, most lack detectable activity toward CDNB, and thus can be missed if GST activity is the only means of GST isolation (Comstock et al. 1994, Lopez et al. 1994). The unique activity of theta-class GSTs can be traced to the presence of the essential Ser-11, responsible for glutathione deprotonation and activation, in place of the tyrosine found in the alpha, mu, and pi class GSTs (Landi 2000). Theta-class GSTs can be further distinguished from alpha/mu/pi class isoforms because of their high affinity for glutathione (high K_m), but low affinity for glutathione-conjugates. The diminished product retention in the active site of theta GSTs favors increased substrate turnover in comparison to alpha/mu/pi forms, which have a greater capacity to sequester conjugated products (Landi 2000). Generally, it is believed that the theta-class GSTs gave rise to the alpha/mu/pi classes via gene duplication events (Pemble & Taylor 1992); however, this has been called into question more recently (Blanchette et al. 2007). In this study, I identified a theta-like GST from *C. gibbosum* digestive gland and by manual validation confirmed the sequence of five peptides to be identical to theta and theta-class related GSTs from fish. The HPLC fraction B shows a single peak, suggesting that only one subunit is expressed. Attempts to amplify theta-like GSTs from *C. gibbosum* digestive gland mRNA with

degenerate primers (not shown) designed to these peptide sequences have not yet succeeded.

It is interesting to note that *Cyphoma* theta-like peptides matched only theta-class GSTs from fish rather than those from terrestrial invertebrates. Using phylogenetic relationships inferred from Bayesian analyses, Lee et al. (2006) found that theta class GSTs formed two distinct, well-supported clades, one ("theta A") containing only fish representatives, which includes the related rho-class GSTs, and the other ("theta B") including theta-class GSTs from mammals, fish, birds, and invertebrates. Recent evidence has confirmed the deeply rooted nature of the "theta A" clade by the identification of a rho-GST in a primitive cephalochordate (Fan et al. 2007) once thought to be a fish-specific class (Lee et al. 2006). The *Cyphoma* GST theta-like peptides matched both rho- and theta-class fish sequences in the "theta A" clade, providing the first evidence for invertebrate members within group.

Several natural compounds, including alpha-tocopherol, coumarin, and indole-3-carbinol, have been identified as potent inducers of mammalian theta GSTs. However, it is the dehalogenase activity of theta-class GSTs that makes this class of enzymes so unique (Landi 2000). For example, both bacterial and mammalian theta-class GSTs are capable of metabolizing dichloromethane (DCM) to formaldehyde (Gisi et al. 1999). The oceans are the largest source of organohalogens (Gribble 2003), with representatives from cnidarians including briarane diterpenes (Kubota et al. 2006), bromo-, chloro- and indo-vulones, clavulones and punaglandins (related to mammalian prostaglandins) (Baker & Scheuer 1994, Rezanka & Dembitsky 2003, Rowley et al. 2005), and chlorinated

sterols (Iwashima et al. 2001). It is possible that *C. gibbosum* theta-like GSTs may have evolved to protect this consumer against halogenated compounds from its gorgonian host.

Numerous studies have begun to identify allelochemical substrates for invertebrate GSTs (reviewed in Li et al. 2007). Many plant allelochemicals and marine natural products contain the appropriate functional groups that can be directly conjugated with GSH (Schlenk & Buhler 1988, Brattsten 1992). *Cyphoma gibbosum* regularly feeds on the gorgonian *Plexaura homomalla,* which contains high concentrations (up to 8% of the dry weight) of prostaglandins (15(R)-PGA_2) (Weinheimer & Spraggins 1969, Dominguez et al. 1980). Prostaglandins are known to suppress cell proliferation and overexpression of mu-class GSTs resulting in increased conjugation of prostaglandins may inhibit the antiproliferative effects of these compounds (Tsuchilda & Sato 1992). Naturally occurring prostaglandins (PGA_2 and PGJ_2) undergo enzymatic conjugation by purified human GSTM1a-1a (Bogaards et al. 1997). Thus, high expression of GST mu isoforms may allow *C. gibbosum* to tolerate the chemical defenses of its host gorgonian and subsequently feed longer than would otherwise be possible. Preliminary studies have demonstrated that prostaglandins found in gorgonians significantly inhibit *Cyphoma* GST activity *in vitro* (Chapter 4), consistent with the idea that gorgonian prostaglandins may be substrates for *Cyphoma* GSTs.

In summary, a proteomic approach was successful at identifying peptides representing the collection of expressed GST subunits in *C. gibbosum* digestive gland. This technique is a valuable alternative to designing degenerate primers to all of the GST classes suspected of being expressed in the sample. Using proteomics, I was able to identify several mu-class GSTs and one theta-class GST subunit(s), the latter class

identified for the first time in a mollusc. The manually validated GST peptides were used to design primers to amplify two full-length *C. gibbosum* mu-class GST cDNAs. Future studies will focus on identifying gorgonian allelochemicals that are substrates for *C. gibbosum* GSTs using a bioassay-guided fractionation approach; expression of recombinant proteins will allow further characterization of individual GST isoforms. The high GST activity and diversity of GST isoforms in *C. gibbosum* may protect this generalist predator against dietary chemicals while conferring a selective advantage over other consumers that avoid chemically defended prey.

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Table 1. Primers used in present study

Gene	Primers	Direction	Sequences (5' to 3')	Purpose
GST Mu	GSTm_R10	Forward	AAY TTR TCR CTI GCC ATR TAI GC	Degenerate, 5'RACE primer
	RACE_F1	Reverse	AAT ACG ACT CAC TAT AGG	Adaptor RACE primer
CgGSTM1	GSTm_F13	Forward	CGC TCA AGC AGA CGT GAT TTG TTG ATA C	Full-length cloning
	AP1	Reverse	CCA TCC TAA TAC GAC TCA CTA TAG GGC	
CgGSTM2	GSTm_F15	Forward	GCT GCG TTT TCT TCT GAT AAC CAA GTC ACT C	Full-length cloning
	AP1	Reverse	CCA TCC TAA TAC GAC TCA CTA TAG GGC	

Table 2. Specific activities and protein fold purification from a representative GST purification from *Cyphoma gibbosum* digestive gland

Fraction	Volume (mL)	Protein (mg/mL)	GST Activity (U/mL)	Total Activity (U)	Specific Activity (U/mg)	Yield	Fold
Cytosol	1.8	7.65	4.36	7.85	0.57	100%	1
G-25 Column						41%	32
Fraction 3	1.0	0.03	0.15	0.15	5.06		
4	1.0	0.70	1.15	1.15	1.64		
5	1.0	0.58	1.00	1.00	1.72		
6	1.0	0.16	0.61	0.61	3.80		
7	1.0	0.05	0.30	0.30	6.00		
GSH-agarose						44%	220
Fraction 18	1.1	0.010	0.28	0.31	28.2		
19	1.1	0.036	2.74	2.97	75.0		
20	0.9	0.010	0.23	0.21	23.3		

Fractions shown here correspond to the same fractions in Figure 1. Units are expressed as μmol/min.

Table 3. Homologous sequences identified from the partial tryptic digests of affinity-purified GSTs separated by HPLC

HPLC fraction or Affinity band	Matched sequence	Class	Reference Species	NCBI Accession #
D, E, Fa, Fb, H, Affinity band 1, 2	GLAQPIR	Mu	Abalone	ABF67506
E, H	GLAQPIRLLLK	Mu	Abalone	ABF67506
C, D, E, Fa, Fb, G, H, Affinity band 1, 2, 3	ITQSNAILR†	Mu	Abalone	ABF67506
G	ITQSNAILRYIAR	Mu	Abalone	ABF67506
Affinity band 3	KAAYFEALPAK	Mu	Abalone	ABF67506
G	SFLGDQQFFAGSK	Mu	Abalone	ABF67506
C, E	IMQPGSLDAFPTLLAFMGRIEALPAIK	Mu	Abalone	ABF67506
Affinity band 1	IEALPAIKTFMSSAK	Mu	Abalone	ABF67506
Fa, Affinity band 2	FIRRPINNK	Mu	Abalone	ABF67506
D	RPINNKSALFK	Mu	Abalone	ABF67506
F	GLGQPIR	Mu	Pacific oyster	CAD90167
F	AYMASDK†	Mu	Pacific oyster	CAD90167
B	SQEVMDMNPR†	Theta	Bass	AAQ91198
B	MFEGLTLNQK†	Theta	Bass	AAQ91198
B	VMIALLEEK†	Theta	Gilt Seabream	AAQ56182
B	VLNESYAACMYLESQFK†	Theta	Plaice	CAA64495
B	LWEGYLQK	Theta	Red Seabream, Bass	BAD98443, AAQ91198
B	GQLPAFK	Theta	Gilt Seabream, Bass	AAQ56182, BAD98443
			Red Seabream	AAQ91198
B	MADVIYYNWK†	Theta	Gilt Seabream, Bass	AAQ56182, BAD98443
			Red Seabream	AAQ91198
B	FGLCEER	Theta	Gilt Seabream, Bass	AAQ56182, BAD98443
			Red Seabream	AAQ91198

†Indicates sequences identical to those of *Cyphoma gibbosum* peptides determined by manual validation.
The HPLC fraction Fa corresponds to the lighter band, and Fb the darker band for fraction F in Figure 4.

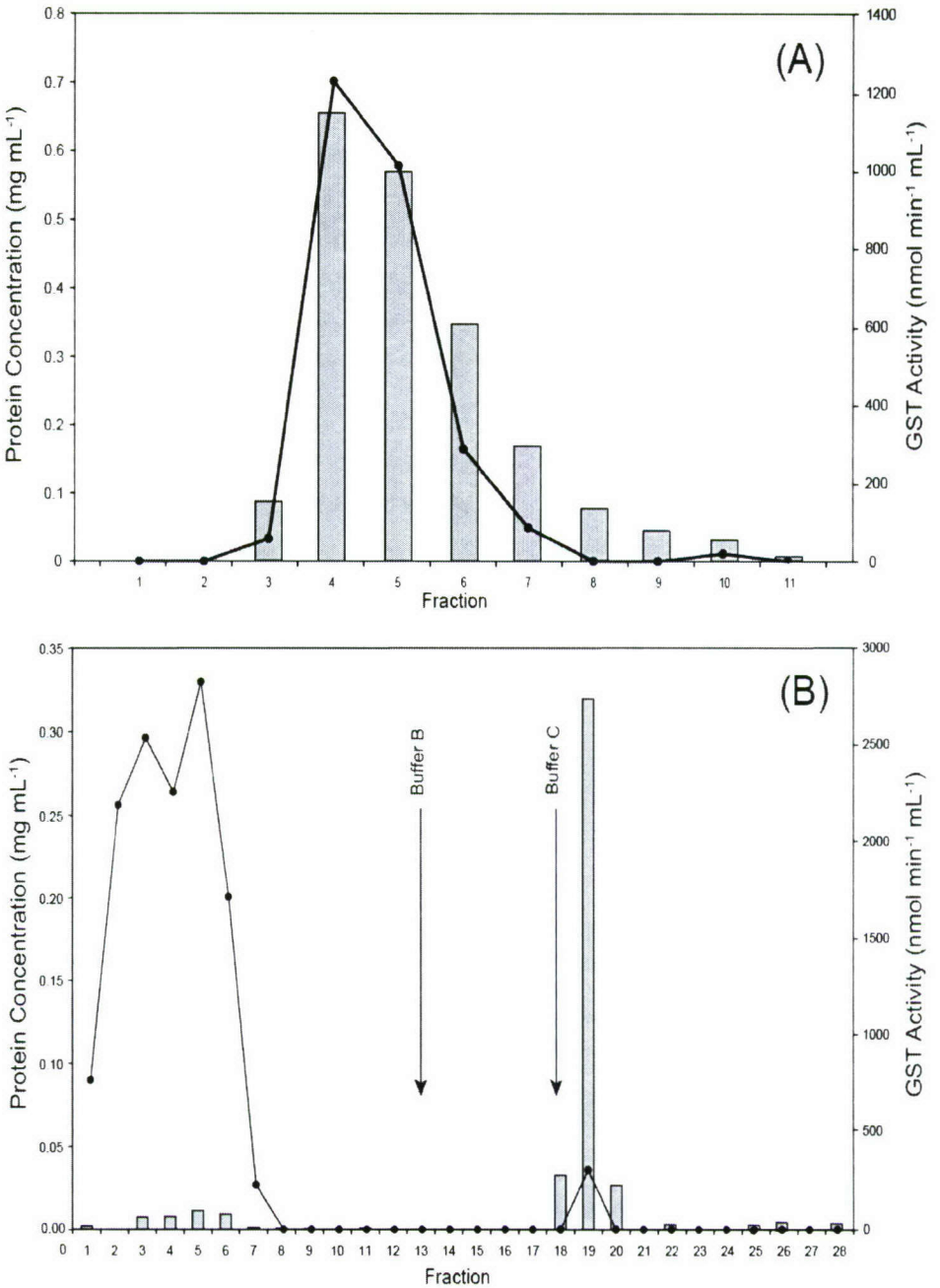


Figure 1. Purification of cytosolic GSTs from *C. gibbosum* digestive gland. (A) The crude cytosol (2.5 mL) from one individual was applied to a size exclusion PD-10 desalting column (bed volume 8.3 mL, 5 cm height) containing Sephadex G-25 matrix (85 – 260 μm) equilibrated in 50 mM Tris buffer, 1mM EDTA, 1mM DTT at pH 6.0 (Buffer A). Fractions were collected in 1.2 mL aliquots by gravity at a flow rate of 1 mL min^{-1}. Fractions 3 – 7 were pooled for further purification. **(B)** The pooled fractions (approx. 6 mL) from the size exclusion column were applied to the GSH-agarose (sulfur to epoxide-activiated 4% cross-lined beaded agarose) affinity column (bed volume 0.5 mL, 0.8 x 4 cm i.d.) equilibrated in Buffer A. The affinity column was first rinsed with 6 mL of Buffer A and then 7 mL of Buffer B (Buffer A + 0.5 M NaCl) to rinse away non-specific proteins. GSTs were eluted with Buffer C (Buffer B + 50 mM GSH, pH 9.5) in 1 mL aliquots at a flow rate of 0.5 mL min^{-1}. Fractions 18 -20 were collected and pooled for further separation by HPLC. GST activity (bars) was measured as the increase in absorbance at 340 nm and protein concentration (•) was estimated by absorbance at 595 nm.

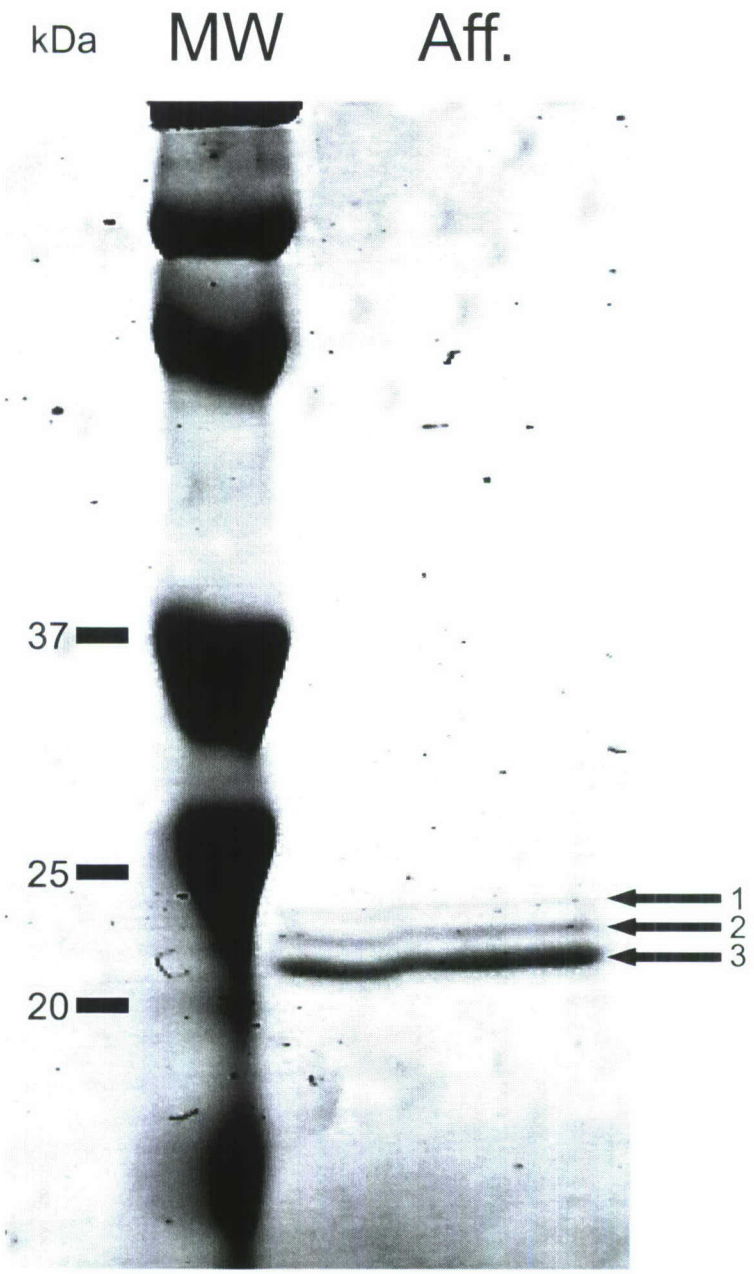


Figure 2. SDS-PAGE of affinity purified GSTs from *C. gibbosum* digestive gland
SDS-PAGE of affinity-purified extract from *C. gibbosum* digestive gland obtained from size exclusion and affinity chromatography. Lane M, Bio-Rad Kaleidoscope protein standards; lane Aff., affinity-purified extract. Bands visualized by silver staining.

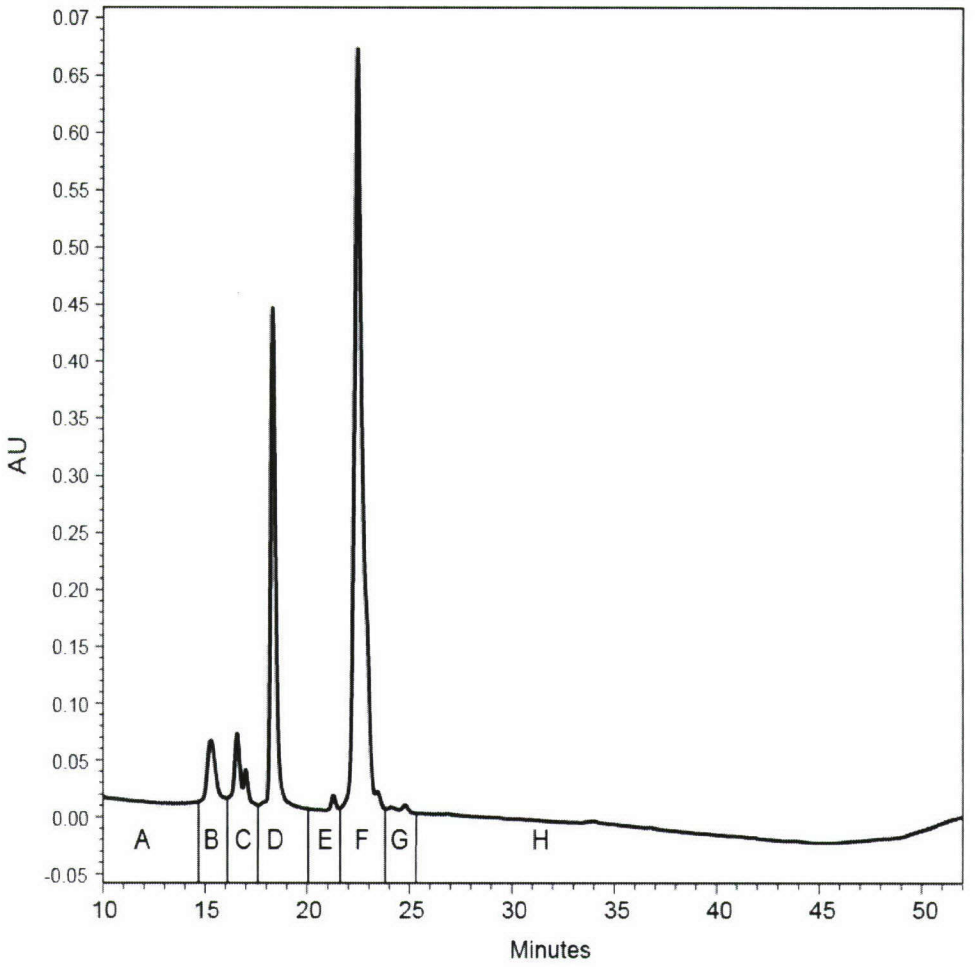


Figure 3. HPLC spectrum of GST subunits. Representative HPLC spectrum of pooled affinity-purified extracts from *C. gibbosum* feeding on *B. asbestinum*. GST subunits were separated on a reverse phase VYDAC protein/peptide column (C18 µm 250 mm x 2.1 mm) with a flow rate of 0.5 mL min^{-1}. Mobile phase A consisted of acetonitrile/water/TFA (38:62:0.1, v/v) and mobile phase B consisted of acetonitrile/water/TFA (80:20:0.1, v/v). GST subunits were separated using a linear gradient from 0 to 40% B in 22 min, and 40 to 100% B in 37 min and visualized at 214 nm. Fourteen unique peaks were identified (not all visible in representative HPLC spectrum) and grouped into fractions B-H. Fraction A consisted of the injection peak (not shown); fraction B was identified as a theta-like GST; fractions C – H were identified as mu-class GSTs.



Figure 4. SDS-PAGE of HPLC separated GST subunits. Seven affinity extracts, pooled by diet, were separated by HPLC and fractions were hand-collected and subject to SDS-PAGE. For simplicity, only HPLC fractions from a single snail diet are shown. Note the appearance of two bands for HPLC fraction F. Bands were visualized by silver staining. The figure is composed of five gels. The same molecular weight ladder and affinity-purified GST fraction were used as standards on each gel. MW: molecular weight standard; Aff: affinity-purified extract (see Figure 2); B-H: hand-collected HPLC fractions.

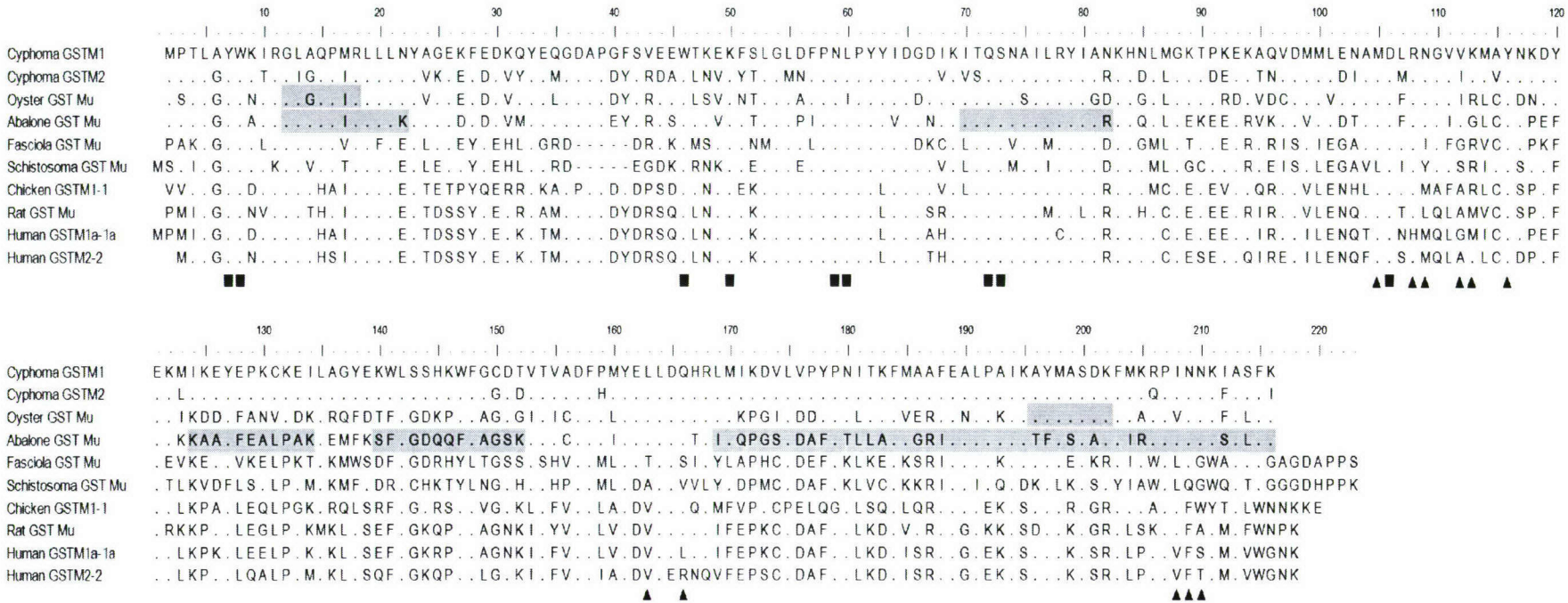


Figure 5. Multiple alignment of deduced amino acid sequences of CgGSTM1 and CgGSTM2 with homologous peptide fragment identification by LC-MS/MS. Comparison of translated mu-class GST cDNA sequences from *Cyphoma gibbosum* with selected vertebrate and invertebrate sequences available in GenBank®. Amino acid residues identical to CgGSTM1 are indicated by a dot. Squares (■) and triangles (▲) indicate residues defining the G-site (GSH binding) and the H-site (substrate binding) respectively, as reported by NCBI Conserved Domain Database (Marchler-Bauer et al. 2007). Shaded boxes represent peptide sequences with homology to tryptically digested peptides from HPLC fractions and affinity-purified bands identified by searching the NCBI protein nrDB (refer to Table 3). The alignment was constructed with the Clustal W algorithm and contains the following Genbank® sequences: EU008562 and EU008563 (*Cyphoma gibbosum*), ABF67506 (abalone), CAD90167 (Pacific oyster), 1XW5B and 1XWKA (human), 1B4PA (rat), 1GSUB (chicken), 1FHE (liver fluke), 1UA5A (blood fluke).

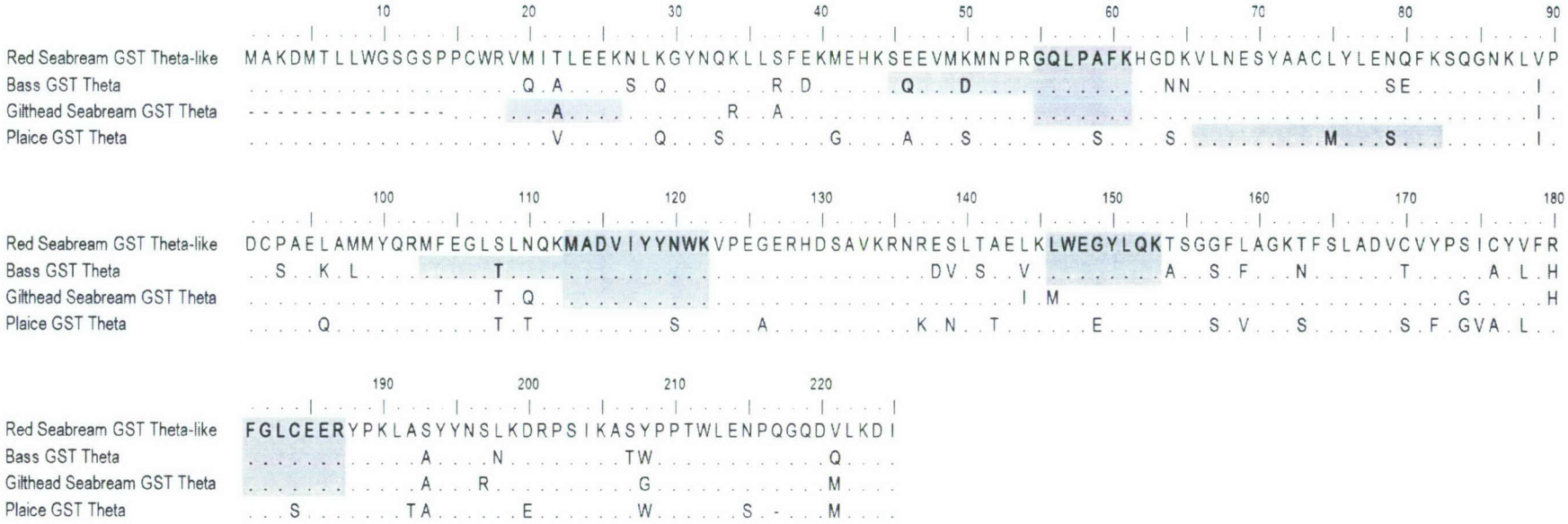
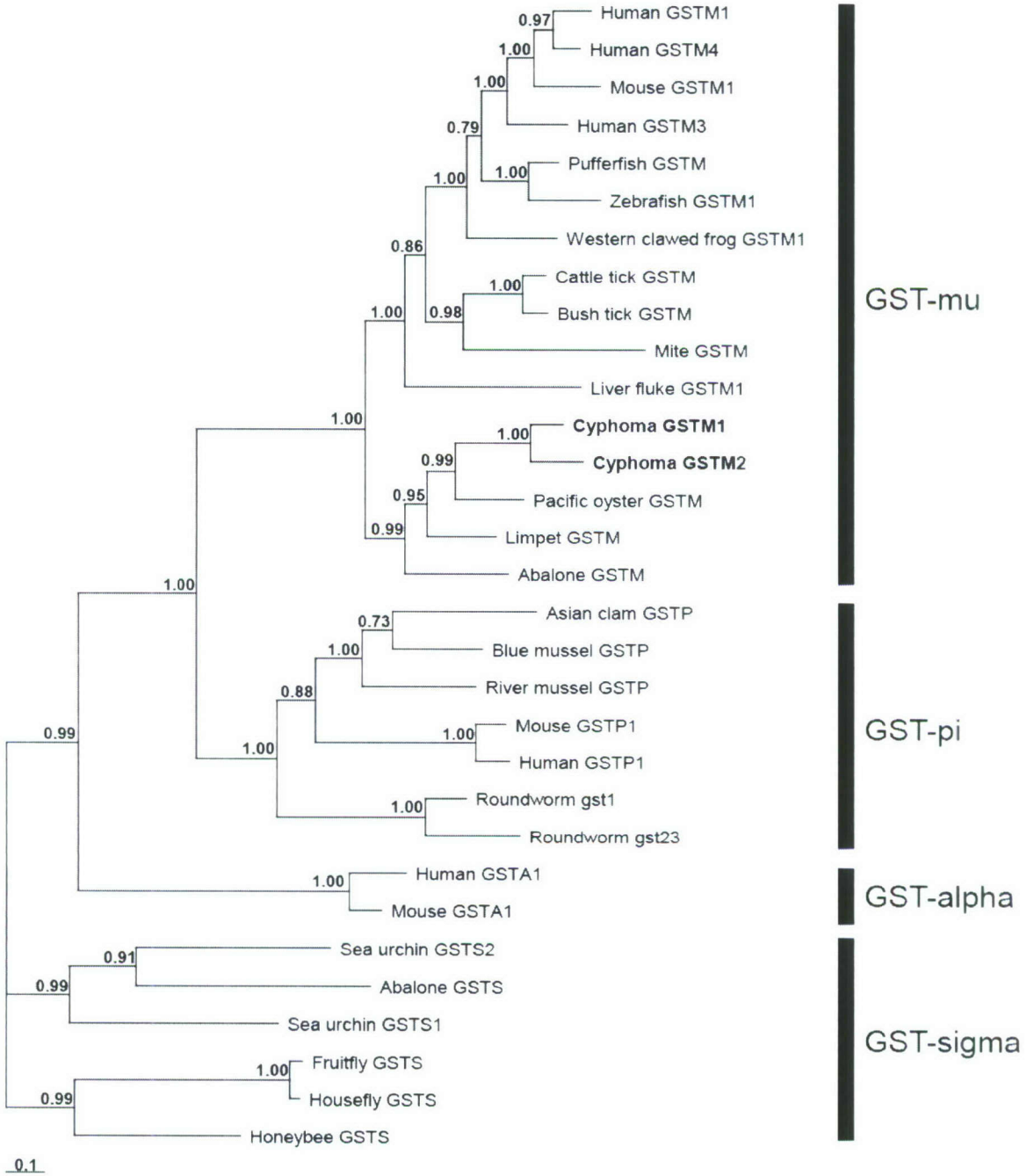


Figure 6. Multiple alignment of theta-class GSTs from fish species with homologous peptide fragment identification by LC-MS/MS. Homology of trypically digested peptides from HPLC fraction B compared with published fish GSTs. Amino acid residues identical in all four fish species are indicated by a dot. Shaded boxes represent peptide sequences with homology to tryptically digested peptides from HPLC fraction B identified by searching the NCBI protein nrDB (refer to Table 3). The alignment was constructed using Clustal W and contains the following Genbank® sequences: BAD98442 (red seabream), AAQ91198 (bass), AAQ56182 (gilthead seabream), and CAA64495 (plaice).

Figure 7. Phylogenetic tree depicting the relationship of *Cyphoma gibbosum* mu-class GSTs with other invertebrate and vertebrate GSTs.
Tree was constructed using MrBayes. Fruitfly, Housefly and Honeybee sigma-GSTs were defined as an outgroup. Values at branch points are posterior probabilities derived from 3e6 generations (see methods). Note the clustering of *Cyphoma* GSTMs (bold) with other molluscan GSTMs. The tree contains the following JGI and Genbank accession nos: *Apis mellifera* (XP_624682), *Boophilus microplus* (AAD15991), *Caenorhabditis elegans* (NP_499006, NP_503889), *Corbicula fluminea* (AAX20374), *Crassostrea gigas* (CAD90167), *Cyphoma gibbosum* (EU008563 and EU008562), *Danio rerio* (XP_690427), *Drosophila melanogaster* (NP_725653), *Fasciola hepatica* (P56598), *Haliotis discus discus* (ABF67506, ABF67507), *Haemaphysalis longicornis* (AAQ74441), *Homo sapiens* (NP_665683, AAV38750, NP_000840, NP_000841, NP_000843), *Lottia gigantea* (JGI scaffold_43000011), *Monodelphis domestica* (P46437), *Mytilus edulis* (AAS60226), *Mus musculus* (P13745, NP_038569, NP_034488), *Strongylocentrotus purpuratus* (XP_795664, XP_785553), *Sarcoptes scabiei* (AAO15607), *Tetraodon nigroviridis* (CAF91521), *Unio tumidus* (AAX20373), *Xenopus tropicalis* (NP_001004964). Tree construction performed by J. Goldstone.


Human GSTM1
Human GSTM4
Mouse GSTM1
Human GSTM3
Pufferfish GSTM
Zebrafish GSTM1
Western clawed frog GSTM1
Cattle tick GSTM
Bush tick GSTM
Mite GSTM
Liver fluke GSTM1
Cyphoma GSTM1
Cyphoma GSTM2
Pacific oyster GSTM
Limpet GSTM
Abalone GSTM
Asian clam GSTP
Blue mussel GSTP
River mussel GSTP
Mouse GSTP1
Human GSTP1
Roundworm gst1
Roundworm gst23
Human GSTA1
Mouse GSTA1
Sea urchin GSTS2
Abalone GSTS
Sea urchin GSTS1
Fruitfly GSTS
Housefly GSTS
Honeybee GSTS
0.1
GST-mu
GST-pi
GST-alpha
GST-sigma

Chapter III

Gorgonian host-inducible cytochrome P450s from *Cyphoma gibbosum*: molecular cloning, diversity and function

Abstract

The production of chemical defenses by marine organisms limits predation and herbivory by most potential consumers. However, there are some marine consumers that can tolerate allelochemicals produced by their prey/hosts seemingly without deleterious effects. The induction of biochemical resistance mechanisms, such as cytochrome P450s (P450s), have been proposed to mediate allelochemical detoxification in these consumers, yet compelling evidence for these claims are generally lacking. Here, I use the generalist marine gastropod *Cyphoma gibbosum*, which feeds exclusively on chemical defended gorgonians, as a model to investigate the diversity, transcriptional response and enzymatic activity of putative allelochemical detoxification P450s in marine consumers. Using an adaptor-ligated cDNA library combined with specific and degenerate primers targeted to cytochrome P450 (CYP) Family 4, twelve new CYP4 genes were identified from the digestive gland of *C. gibbosum*. In controlled laboratory-based feeding studies with *C. gibbosum,* a 2.7- to 5.1-fold induction of CYP4-2 (CYP4BK) and CYP4-3 (CYP4BL) transcripts was observed following dietary exposure to the gorgonian *Plexaura homomalla.* This gorgonian species is known to contain high concentrations of ichthyodeterrent prostaglandins. Phylogenetic analysis reveals *C. gibbosum* CYP4-2 and CYP4-3 closest vertebrate relatives include CYP4A and CYP4F genes, whose roles entail metabolic inactivation of pathophysiologically important fatty acid derivatives, including prostaglandins. Examination of fatty acid hydroxylase activity in *Cyphoma* microsomes and allelochemically-responsive recombinant *Cyphoma* P450s indicates a possible role in eicosanoid (e.g., leukotriene B_4) metabolism. Sequence analysis further demonstrates that *C. gibbosum* CYP4s share identical amino acid residues with vertebrate 4A and 4F

forms at key positions within the active site or sites responsible for maintaining the correct orientation of the fatty acid within the access channel, further alluding to their possible role in prostaglandin metabolism. These results are the first to describe the differential regulation of specific P450 transcripts in a marine consumer in response to a diet rich in allelochemicals. This work has important implications for understanding the molecular basis of the biochemical resistance strategy marine consumers use to cope with their chemically defended prey, and the ancestral history and evolutionary progression of allelochemical metabolizing genes within the P450 superfamily.

Introduction

Gorgonian corals (Phylum: Cnidaria; Subclass: Octocorallia; Order Gorgonacea) are the most conspicuous group of invertebrates on Caribbean coral reefs (Goldberg 1973, Lasker & Coffroth 1983); however, they are rarely consumed despite their abundance and the intense predation in these ecosystems (O'Neal & Pawlik 2002). The dominance of gorgonians can be attributed, in part, to the variety of defensive secondary metabolites they produce, commonly referred to as allelochemicals (Pawlik et al. 1987, O'Neal & Pawlik 2002, Paul & Puglisi 2004). An allelochemical is a "chemical produced by an organism that is toxic to, or inhibits the growth of, other organisms" (Despres et al. 2007). Octocorals, in particular, produce a diverse array of terpenoid derivatives that have been shown to have antipredatory properties (Rodriguez 1995, Katz & Adamczeski 2000, Paul & Puglisi 2004). Although these chemical defenses limit the predation by most potential consumers, the ovulid gastropod *Cyphoma gibbosum* thrives solely on a diet of allelochemically-rich octocorals and is considered the principle consumer of

gorgonians on Caribbean coral reefs (Birkeland & Gregory 1975, Burkepile & Hay 2007). High levels of predation by this mollusc have also been shown to significantly increase production of the ichthyodeterrent terpenoids in *in situ* caging studies (Thornton & Kerr 2002). The mechanisms by which *C. gibbosum* can tolerate these and other dietary allelochemicals at natural or elevated concentrations are unknown.

A wealth of literature describes the diversity of gorgonian allelochemicals and their effects on consumers (Gerhart 1984, Pawlik et al. 1987, Harvell et al. 1988, Pawlik & Fenical 1989, Fenical & Pawlik 1991, Pawlik & Fenical 1992, Gerhart & Coll 1993, O'Neal & Pawlik 2002; reviewed in Harper et al. 2001); yet, relatively little is known about the mechanisms marine consumers have evolved to overcome the chemical defenses of their hosts/prey. While avoidance of toxic prey (Gerhart 1991, Long & Hay 2006), diet-mixing (Harvell & Suchanek 1987), sequestration (Proksch 1994, Cimino & Ghiselin 1999) and target-site mutation conferring insensitivity (Bricelj et al. 2005) have been described in marine systems, few studies have investigated allelochemical detoxification as a mechanism by which marine consumers may use to cope with noxious dietary compounds.

In comparison to consumer-prey dynamics in marine ecosystems, insect-plant allelochemical interactions are better understood and provide a conceptual framework for investigating how marine consumers respond to toxins both on a behavioral and cellular/biochemical level. In terrestrial ecosystems, metabolic resistance to host plant toxins is attributed, in part, to an arsenal of inducible detoxification enzymes termed cytochrome P450 monooxygenases (P450s), capable of protecting the consumer from dietary intoxication (Schuler 1996). Furthermore, much of the apparent diversity of P450

isoforms in insects may be the result of the co-evolutionary 'arms race' between prey chemical defenses and predator detoxification mechanisms (Gonzalez & Nebert 1990). According to this view, multiple duplication and divergence events among P450 genes have allowed isoforms to gain new functions and presumably to increase the pool of allelochemical substrates upon which they act (Li et al. 2003), while still allowing some isoforms to retain their ancestral metabolic capabilities (Li et al. 2002a). A similar diversification of P450s genes may have occurred in marine invertebrates feeding on chemically-defended prey, providing consumers like *C. gibbosum* with protection against the continuous barrage of defensive compounds they may encounter.

Vrolijk and Targett (Vrolijk & Targett 1992) were the first to measure P450 content and activity from field-collected *C. gibbosum* feeding on four species of gorgonian corals (*Briareum asbestinum, Gorgonian ventalina, Plexaura homomalla*, and *Pseudopterogorgia americana*). The specific content of cytochrome P450 in the digestive gland was low and only quantifiable in *C. gibbosum* collected from *P. americana*. The authors suggested that the low specific content may have resulted from denatured P450 protein, which was confirmed by the presence of 420 nm peaks in the CO-difference spectra. Additionally, two spectrofluorometric assays (methoxyresorufin O-deethylase (MROD) and ethoxyresorufin O-deethylase (EROD)) used to detect P450 activity in *C. gibbosum* digestive gland microsomes were negative. MROD and EROD assays have classically been used to detect subfamily specific P450 activity (i.e. CYP1A) in vertebrates exposed to pollutants (e.g. planar halogenated and polycyclic aromatic hydrocarbons) (Stegeman & Hahn 1994). While evidence of deuterostome (e.g. tunicate and sea urchin) CYP1A-like and CYP1-like genes exists (Goldstone et al. 2007), to date

no full-length CYP1-like sequences have been reported for molluscs (Rewitz et al. 2006) or any other protostome, further suggesting that the CYP1 family may be deuterostome specific. Recent work by Grosvik et al. (2006) strengthens this argument by calling into question immunochemical evidence suggesting the presence of a CYP1A-like protein in molluscs. Therefore, it is not entirely surprising that *C. gibbosum* digestive gland tissues lack CYP1A-like enzyme activity, which does not preclude the possibility of other xenobiotic detoxifying CYPs being responsible for allelochemical resistance.

Members of the P450 (CYP) family 4 represent a substantial portion of the cDNA sequences identified to date in molluscan species (Snyder 1998, Chaty et al. 2004). This family is considered one of the most ancient P450 families, having evolved from the steroid-synthesizing P450s and since diverged into an array of subfamilies whose substrates may be more diverse than previously realized. In vertebrates, CYP4 genes are recognized as the predominant fatty acid ω-hydroxylases, preventing lipotoxicity by hydroxylating eicosanoids, including prostaglandins (Kikuta et al. 2002, Hsu et al. 2007). Prostaglandins are potent signaling molecules, known as regulators of fever, inflammation, and pain response in human biology. In marine systems, prostaglandins are known to function as feeding deterrents and can comprise up to 8% of the dry weight of some gorgonian species (reviewed by Stanley 2000). Yet the diversity of CYP4 substrates extends beyond fatty acid derivatives.

The ability of P450s to metabolize terpenes is of particular interest here due to the predominance of both diterpenoid and sesquiterpenoid compounds across all members of the Octocorallia (Coll 1992). Terpene derivatives, including the sesquiterpene farnesol as well as sesquiterpenoid epoxide juvenile hormone III, are

substrates for the cockroach CYP4C7 (Sutherland et al. 1998, Sutherland et al. 2000). CYP4D10 isolated from the Sonoran Desert endemic *Drosophilia mettleri* was significantly induced in response to exposure to defensive isoquinoline alkaloids specific to its host the saguaro cactus, suggesting that this CYP may be responsible for their detoxification (Danielson et al. 1998). Allelochemical resistance in this species of insect may also involve cooperative action among multiple P450 isoforms, possibility within the same family, allowing this generalist to exploit a broader range of plants with structurally similar defensive compounds. Evidence from these and other studies of insect allelochemical metabolism highlight the relative importance of both P450 diversification and modulation of transcript expression in explaining consumer patterns of host plant utilization. Whether *C. gibbosum* possesses a similar diversity of allelochemically responsive CYP4 genes is the focus of this investigation.

Vrolijk and Targett (1992) originally hypothesized that the ability of *C. gibbosum* to tolerate dietary allelochemicals may involve biochemical resistance mechanisms, such as inducible cytochrome P450s. Here, I explore this hypothesis further by using a molecular approach to identify CYP4 genes in *C. gibbosum* and examine their transcriptional response following exposure to variety of gorgonian allelochemicals in a series of feeding assays. Once allelochemically-responsive CYP4 genes were identified, representative forms were heterologously expressed and their ability to metabolize diagnostic substrates was examined. Evidence presented here emphasizes the role of P450 genes in the adaptation of marine consumers to their chemically defended prey.

Materials and Methods

Animal collection and feeding assay design

A total of 151 adult *Cyphoma gibbosum* (ca 2-3 cm length) were collected from five shallow reefs (< 20m) (Big Point – 23°47.383'N, 76°8.113'W; North Normans – 23°47.383'N, 76°8.264'W; Rainbow Gardens – 23°47.792'N, 76°8.787'W; Shark Rock – 23°45.075'N, 76°7.475'W; Sugar Blue Holes – 23°41.910'N, 76°0.23'W) near the Perry Institute of Marine Science (PIMS), Lee Stocking Island, Exuma Cays, Bahamas (Figure 1) in January 2006. Snails were immediately transported to wet laboratory facilities provided by PIMS where a series of feeding assays were conducted with seven gorgonian species (*Briareum asbestinum, Eunicea mammosa, Gorgonia ventalina, Pseudopterogorgia acerosa, Pseudopterogorgia americana, Pseudopterogorgia elisabethae, Plexaura homomalla*) observed to serve as hosts for *C. gibbosum* in the field. Individual snails were housed separately in 3-L polycarbonate tanks which were placed in a 12' x 20" raceway supplied with filtered, continuous-flow seawater. This design allowed for a common water source to feed each tank but prevented mixing between tanks. Snails collected from the same reefs were housed separately in the same raceways.

Snails collected from each reef were randomly assigned to one of nine groups: one of seven gorgonian diets, a control diet, or a time-zero group at the start of the feeding assays. Snails in the time zero groups (n = 6 or 7 snails/reef location) were dissected within two hours after field collection and digestive glands were preserved in RNA Later® (Ambion) and stored at -80°C. These time-zero snails provide baseline information about the CYPs expressed in a population of *C. gibbosum* on a particular reef at the time of collection. The remaining snails were included in the feeding assay and

maintained on their respective diet (gorgonian or control) for a total of four days. A single feeding assay consisted of two raceways, each holding ten tanks - seven tanks containing one *C. gibbosum* feeding on one of seven gorgonian diets and three tanks each containing one *C. gibbosum* feeding on the control diet. This design accommodated 20 snails feeding at any one time. Snails within each raceway were collected from the same reef and each four-day feeding assay (2 raceways) was repeated a total of six times (Figure 2). Upon completion of the 4 d feeding assay, the digestive glands were immediately dissected, weighed, preserved in RNA Later® at -80°C for real-time quantitative PCR expression analysis and transported back to the Woods Hole Oceanographic Institution. Tissues were maintained at -80°C until further analysis.

Running in parallel with the first set of feeding assays was a second set of feeding assays. Digestive gland tissues collected during the second feeding assays were used to investigate enzymatic activity of *C. gibbosum* digestive gland microsomes (described below) and cytosolic fractions (described in Chapter 2). The design of the second feeding assay was exactly as described above, except one raceway with ten tanks each holding a single snail was used. Snails within the raceway were collected from the same reef, and each 4 d assay was repeated four times (Figure 3). A total of 39 snails were collected from four reefs (Big Point, Rainbow Gardens, Shark Rock and Sugar Blue Holes) and randomly assigned to either a control diet or one of six gorgonian diets (*B. asbestinum, E. mammosa, G. ventalina, P. acerosa, P. americana, P. homomalla*). Following the completion of the 4 d feeding assay digestive glands were immediately dissected, weighed, and frozen in liquid nitrogen and transported to the Woods Hole Oceanographic Institution. Tissues were maintained at -80°C until further analysis.

Table 1 provides a summary of digestive gland samples collected from both feeding assays and processed for RNA and protein analysis.

A minimum of ten colonies for each gorgonian species were collected from shallow reefs (< 20m) surrounding PIMS and housed in separate raceways prior to introduction into the tanks containing *C. gibbosum*. The maximum amount of time between gorgonian field collection and introduction into the feeding assay was 12 hours. Gorgonian colonies were cut into 2-3 inch pieces and allowed to 'heal' for four hours before addition to *C. gibbosum* tanks. The control diet, which closely approximated the nutritional quality of gorgonian tissue, consisted of a combination of alginic acid and freeze-dried squid powder prepared by the method of (O'Neal & Pawlik 2002) with some modification. The squid-alginate paste was pressed into sixteen 3-mm deep wells which had been drilled into a 3" x 1" piece of Formica® resembling a domino. The domino was then placed into a 0.25 M calcium chloride solution allowing the squid-aliginate paste to harden. Theoretically, snails feeding on the control diet devoid of gorgonian compounds should experience no induction of allelochemically-responsive CYPs. Both control and gorgonian diets were replaced each day for four days and feeding activity was monitored by the presence of feeding scars on their gorgonian prey and empty wells on control dominos.

The length (4 d) of the feeding assay was decided based upon previous studies that measured *C. gibbosum* residence time on gorgonian colonies (Harvell & Suchanek 1987) and known detoxification enzyme induction times in other invertebrates (DeBusk et al. 2000, Li et al. 2000, Li et al. 2002c, Kuhajek & Schlenk 2003, Rewitz et al. 2004) (David et al. 2006). Studies examining the half lives of CYP mRNA have found that

most decline rapidly within 24 hours (Daujat et al. 1991, Kloepper-Sams & Stegeman 1994, Lekas et al. 2000) – some as little as two hours in terrestrial invertebrates (Sutherland et al. 2000) when the chemical stimulus is removed. Therefore, any CYP mRNA induction we observe in *C. gibbosum* digestive glands should be attributed to the xenobiotics in their most recent diet.

Initial RNA extraction and RT-PCR

In 2004, a preliminary series of feeding assays with 15 adult *C. gibbosum* and four gorgonian species (*Briareum asbestinum, Gorgonia ventalina, Pseudopterogorgia acerosa, Pseudopterogorgia americana*) provided tissue for the initial cloning of CYP4 fragments. Digestive gland samples were stored in RNALater® at -80°C until further processing. Total RNA was isolated from pooled digestive glands using RNA STAT-60 (Tel-Test B, Inc., Friendswood, TX) according to the manufacturer's protocol. The integrity of the RNA sample was checked by electrophoresis on a 1% agarose-formaldehyde gel in MOPS buffer and visualized with ethidium bromide under UV light. Total RNA concentration was determined using a Nanodrop spectrophotometer (Wilmington, DE). Poly(A)+ RNA was purified using the MicroPoly(A)Purist mRNA purification kit (Ambion, Austin, TX) according to the manufacturer's instructions. First-strand cDNA was reverse transcribed from 2 μg poly(A)+ RNA using OmniScript reverse transcriptase (OmniScript RT kit, Qiagen) with random hexamer primers. PCR was performed using AmpliTaq Gold DNA polymerase (Applied Biosystems) under the following conditions: 94°C for 10 min; (94°C for 15 sec, 52°C for 30 sec) for 35 cycles; 72°C for 7 min with pairs of degenerate primers (CYP4_F1/ CYP4_R3), (CYP4_F2/

CYP4_R3) (see Table 2) designed against the conserved CYP4 P450 fingerprint region. Primers were obtained from Sigma Genosys (St. Louis, MO). PCR products were visualized on agarose gels, gel purified (Gene Clean II, Bio 101, Inc.), ligated into pGEM-T Easy© plasmid vector (Promega, Madison, WI), and transformed into JM109 cells (Promega). PCR products were sequenced in both directions using an ABI 3730XL capillary sequencer at the Keck facility of the Josephine Bay Paul Center for Comparative Molecular Biology & Evolution at the Marine Biological Laboratory (Woods Hole, MA). A minimum of twelve clones were sequenced for each PCR fragment. Clones were initially clustered based on nucleotide sequence identity (>80%) with Sequencher 4.6 (Gene Codes Corp., Ann Arbor, MI) and a consensus sequence was generated and examined by NCBI/GenBank BLASTx for gene identification (Altschul et al. 1997).

Rapid amplification of cDNA ends (RACE)

Digestive gland total RNA was purified from seven *C. gibbosum* individuals collected during the January 2006 feeding assays using the RNeasy Maxi Kit and DNAse treated using a RNase-free DNAse Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The integrity and concentration of total RNA was determined as described in Chapter 2, Methods. Poly(A)+ RNA was isolated using the MicroPoly(A) Purist mRNA purification kit according to the manufacturer's instructions. Poly(A)+ RNA was pooled from seven snails feeding either on a control diet or one of six gorgonian species (0.14 µg poly(A)+ RNA/individual) to ensure representation of all CYPs expressed under various dietary conditions. One microgram of pooled

poly(A)+RNA was primed with modified oligo (dT) primers and used to create an adaptor-ligated double-stranded cDNA library synthesized using the Marathon cDNA Amplification Kit (BD Biosciences, Palo Alto, CA) according to the manufacturer's instructions.

Gene specific primers designed to the three partial cDNA sequences (Table 2) obtained in the initial RT-PCR experiment were used in combination with adaptor-specific primers to obtain full-length CYP sequences by 5' and 3' RACE-PCR. Primers were obtained from Sigma Genosys (St. Louis, MO). Amplification of PCR products was carried out according to the Advantage 2 PCR Enzyme Kit (Clontech, Mountain View, CA) and cycling parameters were as follows: 94°C for 30 sec; (94°C for 5 sec, 72°C for 2.5 min) for 5 cycles; (94°C for 5 sec, 70°C for 2.5 min) for 5 cycles; (94°C for 5 sec, 68°C for 2.5 min) for 25 cycles; 68°C for 5 min with the following specific primer pairs (RACE_1_F/ CYP4_A12_R), (RACE_1_F/ CYP4_D09_F), (RACE_1_F/ CYP4_F11_R), (RACE_1_F/ CYP4_F1), and (AP1/ CYP4-3_R1). Once the start and stop codons had been obtained, primers were designed immediately up or down stream, respectively, to amplify full-length cDNA. Full-length products were amplified with PfuUltra™ Fusion HS DNA Polymerase (Stratagene, La Jolla, CA) and cycling parameters were as follows: 95°C for 1 min; (95°C for 20 sec, 63°C for 20 sec, 72°C for 1min) for 40 cycles; 72°C for 3 min with specific primers pairs CYP4-3_F3/ CYP4-3_R6, and CYP4-2_F1/ CYP4-2_R1. All PCR products were sequenced and analyzed as described above.

Sequence analysis and structural alignments

Partial and full-length CYP4 nucleotide sequences were clustered by Sequencher 4.6 based on nucleotide identity (> 80% identity), aligned using ClustalX (Thompson et al. 1994) and this alignment was used to construct maxium parsimony trees using PAUP*4.0b10 (Swofford 1998) from which the number of possible distinct CYP4 loci were inferred. Trees were visualized and manipulated using FigTree v1.1(Rambaut 2007). Sequences within each cluster were then grouped according to maximum parsimony tree results, and consensus nucleotide and deduced amino acid sequences were generated from these groupings in BioEdit v7.0.5.2 (Hall 1999).

Multiple alignments of *Cyphoma* deduced amino acid sequences and other invertebrate and vertebrate full-length CYP4 gene sequences were performed by ClustalX for phylogenetic analysis by Bayesian and maximum likelihood methods. Ambiguous alignment positions were excluded from further analyses. Bayesian phylogenetic relationships were investigated using the computer program MrBayes (v 3.1.2; Ronquist & Huelsenbeck 2003). MrBayes estimates posterior probabilities using Metropolis-Hastings coupled Monte Carlo Markov chains (MC3). MC3 estimates with uninformative prior probabilities were performed using the WAG model of amino acid substitution (Whelan & Goldman 2001) and prior uniform gamma distributions approximated with four categories (WAG+I+gamma). Four incrementally heated, randomly seeded Markov chains were run for $3x10^6$ generations, and topologies were sampled every 100th generation. The MC3 burnin values were conservatively set at $1x10^6$ generations. Posterior probabilities of topologies and clades were estimated from the sampled topologies after removal of the initial MC3 burnin. Maximum likelihood phylogenetic

relationships were calculated with the Pthreads version of RAxML v7.0.0 (Stamatakis 2006, Ott et al. 2007) using the WAG+gamma model of amino acid substitution. Multiple initial ML searches were performed from random starting points and bootstrap support was estimated for the best ML tree.

Identification of putative substrate recognition sites (SRSs) of *Cyphoma* CYPs was accomplished by using the bacterial CYP102 (Ravichandran et al. 1993) as a template to highlight (putative) active site residues. Bacterial CYPs have often been used convincingly as templates for eukaryotic P450 homology modeling studies (Chang et al. 1996). ClustalX was used to align full-length *Cyphoma* deduced amino acid sequences with CYP102 and selective mammalian CYPs whose SRSs have previously been determined (Gotoh 1992, Loughran et al. 2000, Kalsotra et al. 2004). Alignments were visualized in BioEdit v7.0.5.2 (Hall 1999) and structurally conserved regions (e.g., helices and β-structures) and SRS for *Cyphoma* CYPs were generated by copying the backbone coordinates from CYP102.

Real-time quantitative RT-PCR

DNAse-treated poly(A)+ RNA (0.2 μg) from the digestive gland of snails participating in the 2006 feeding assay (n = 141, Table 1) was used to synthesize cDNA using a blend of oligo (dT) and random hexamers as decribed by the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The quantity of poly(A)+RNA for cDNA synthesis was chosen based on an experiment comparing the product yield (e.g. target gene expression) versus input concentration of poly(A)+RNA (Figure 4). PCR reactions were performed using iQ SYBR Green Supermix (Bio-Rad) using an iCycler MyiQ Real-

Time PCR Detection System (Bio-Rad). Sequence-specific primers for quantitative PCR analysis of CYP mRNA expression are listed in Table 3. In addition, a representative group of poly(A)+ RNA samples was used to perform control cDNA synthesis experiments without reverse transcriptase to check for contamination by genomic DNA. The PCR conditions were as follows: 95°C for 3min; (95°C for 15 sec, 64°C or 62°C for 1 min) for 40 cycles. PCR product specificity from each primer pair was confirmed by melt curve analysis to ensure that only a single product was amplified. A standard curve was generated for each CYP grouping by serially diluting plasmids containing the fragment to be amplified. Each sample and standard was run in duplicate and the expression of *C. gibbosum* β-actin was used to control for differences in cDNA synthesis among samples. The molecule number per gene in each RNA sample was calculated from the standard curve.

Data analysis of RT-qPCR experiments

To test whether cytochrome P450 gene expression differed between snails feeding on a control diet versus each gorgonian diet, a two way multivariate analysis of variance (MANOVA) was used with Diet (control vs. gorgonian diet) as a fixed factor and Reef (snail origin) as a random factor. The CYP4 genes were considered dependent variables. If Diet was found to be significant in the two-way MANOVA, univariate two-way ANOVAs were run for each CYP4 gene to determine which diet showed significant differences. In addition, a one-way MANOVA was used to examine the variability in CYP4 gene expression among reefs, where Reef was considered a random factor. This test was used to investigate reef-specific variation in transcript levels in time-zero snails,

and to determine if any such variation persisted in snails collected from these same reefs after being fed a control diet for four days. The CYP4 genes were considered dependent variables.

P-values have been corrected for both MANOVA analyses using Bonferroni's adjustment (Sankoh et al. 1997). Data analysis was performed using SYSTAT® version 11 (Systat Software, Inc., San Jose, CA). Data reported as molecule numbers for each gene were log transformed to homogenize variances for MANOVA and ANOVA analysis.

Heterologous expression of Cyphoma CYPs in yeast

The open reading frame (ORF) of five CYPs, representing a diversity of the CYP4 sequences from *Cyphoma* (CYP4-2a = clone 198_58; CYP4-2b = clone 198_27; CYP4-3a1 = clone 197_52; CYP4-3b1 = clone 197_48; CYP4-3b3 = clone 197_53), were amplified with custom primers (Table 4) and PCR fragments were ligated into pENTR/D/TOPO® according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). PCR products were amplified using PfuUltra™ (Stratagene, La Jolla, CA) with the following conditions: 95°C for 2 min; (95°C for 20 sec, 63.2°C for 20 sec, 72°C for 30 sec) for 30 cycles; and 72°C for 3 min for final extension. Primers were used at 10 μM in a 50 μL reaction. PCR products were separated on a 1% agarose gel and discrete bands were excised and extracted with the Geneclean® kit (Qbiogene, Irvine, CA) according to the manufacturer's protocol.

ORFs for all five clones were transferred from the entry vector pENTR/D/TOPO to the destination vector pYESDEST52/V5-His using the TOPO Cloning Kit (Invitrogen)

and provided protocol. A pYESDEST52 plasmid carrying the *Arabidopsis* β-glucuronidase (gus) gene was used as a transformation and expression control. Expression clone plasmids were purified from overnight *E. coli* culture and used for yeast transformation. *Saccharomyces cerevisiae* W(R) strain, which over-expresses the yeast NADPH-cytochrome P450 reductase under a galactose-inducible promoter, was a kind gift of Drs. D. Pompon and P. Urban (Center de Génétique Moléculaire, CNRS, Gif-sur-Yvette, France) (Pompon et al. 1996). The W(R) yeast was made competent using the *S.c.* EasyComp™ transformation kit according to the manufacturer's instructions (Invitrogen) and separately transformed with the expression clones containing *C. gibbosum* P450s.

To achieve higher recombinant protein expression, yeast cells were initially grown to high density with glucose as the main carbon source; thereafter, galactose was added to induce expression. The production of recombinant CYP protein was as follows: a single transformed yeast colony was picked from a selective plate and used to inoculate 30 mL of SGI medium (containing per liter, 6.7g yeast nitrogen base without amino acids, 1g bactocasamino acids deficient for uracil, 40 mg DL-tryptophan, 20 g glucose, 100 mg adenine). The SGI culture was grown at 28°C with shaking at 130 rpm overnight, then transferred (10 mL) to a 2-liter flask containing 500 mL of YPGE media (containing per liter, 10 g yeast extract, 10 g bactopeptone, 5 g glucose, 100 mg adenine, 3% ethanol). The YPGE culture was grown overnight at 28°C with shaking at 130rpm. Yeast cells were induced by the addition of 2% galactose for 8 or 15 hrs at 28°C with shaking at 130rpm. Cells were harvested by centrifugation, washed with TES50 buffer

(50 mM Tris-HCl, 1 mM EDTA, 0.6 M sorbitol; pH 7.4) and stored at -80°C until microsomal preparation.

Preparation of yeast microsomes

Yeast cells were suspended in degassed TES50 buffer containing 1.0 mM dithiothreitol (DTT) and protease inhibitor cocktail (1X) (Sigma) and mechanically disrupted using the BeadBeater (BioSpec Products, Inc., Bartlesville, OK). A 25 mL chamber containing about 50% glass beads (0.5 mm diameter) and yeast cell slurry was cooled with an ice water/methanol (20%) cooling jacket. Any air remaining in the chamber was displaced by adding TES50 buffer. The cells were disrupted by twenty 5-s BeadBeater cycles with 45-s resting (cooling) periods between cycles. This method of disruption has been shown to be effective at maintaining the sample temperature below 8°C during disruption and results in greater than 90% lysis of yeast cells (Chung et al. 2004).

All subsequent steps were carried out at 4°C. Cell lysate was decanted and the beads remaining in the chamber were washed once with 2 mL of degassed buffer and combined with the cell lysate. The combined cell lysate was centrifuged at 750 x g for 10 min then, without stopping, at 12,000 x g for 10 min using a Beckman J2-21 centrifuge (Fullerton, CA) to remove cell debris. The supernatant was carefully removed and centrifuged at 100,000 x g for 70 min using a Beckman L8-60M ultracentrifuge (Fullerton, CA) to collect the microsomal fraction. The resulting microsomal pellet was resuspended in 1 to 2 mL of TEG50 buffer (50 mM Tris-HCl, 1 mM EDTA, 20% glycerol (by vol.), 1 mM DTT; pH 7.4) by gentle hand homogenization using a Potter-

Elvehjem homogenizer and microsomal suspensions were stored at -80°C until use. An aliquot of suspension was taken for microsomal protein determination using the Bradford assay method (Bradford 1976) with BSA as the standard.

Preparation of Cyphoma digestive gland microsomes

Individual digestive glands (n = 49) were homogenized 1:4 (w/v) in ice-cold homogenization buffer (0.1 M potassium phosphate, 1 mM EDTA, 1 mM DTT, 1.15% potassium chloride, protease inhibitor cocktail (1X); pH 7.5) with an IKA Ultra Turrax T8 homogenizer (Wilmington, NC) for 30 sec on ice. All subsequent steps were carried out at 4°C. Cytosol was isolated by centrifugation of the crude homogenate at 750 x *g* for 10 min then, without stopping, at 12,000 x *g* for 10 min using a Beckman J2-21 centrifuge (Fullerton, CA). The supernatant was carefully removed, avoiding both the fatty layer and the pellet and transferred to an ultracentrifuge tube and centrifuged at 100,000 x *g* for 70 min using a Beckman L8-60M ultracentrifuge (Fullerton, CA). The microsomal pellet was resuspended in 0.2 to 0.8 mL of microsomal buffer (0.1 M potassium phosphate, 1 mM EDTA, 1 mM DTT, 20% glycerol (by vol.); pH 7.5) and stored at -80°C until use. An aliquot of suspension was taken for microsomal protein determination using the Bradford assay method (Bradford 1976) with BSA as the standard.

P450 content and enzymatic assays

Cytochrome P450 specific content of yeast and digestive gland microsomal samples was determined by the carbon monoxide difference spectrum ($\Delta OD_{450\text{-}490}$) of

sodium dithionite-reduced samples (1 mg/mL) using an extinction coefficient of 91 $mM^{-1}cm^{-1}$ by the method of (Omura & Sato 1964). P450 reductase in both yeast and digestive gland microsomal samples was assayed by measuring NADPH-cytochrome *c* reductase activity. The reaction mixture (in a final volume of 200 µL) contained 0.2 M potassium phosphate buffer, pH 7.7, 80 µM equine heart type III Cytochrome *c* (Sigma), and 0.2 mM NADPH. NADPH was solubilized in TEG50 buffer. The rate of Cytochrome *c* reduction was measured as the increase in absorbance at 550 nm ($\Delta\varepsilon_{550}$ 21.1 mM^{-1} cm^{-1}) using a UV-2401PC spectrophotomer (Shimadzu). One unit of reductase activity is defined as the amount of enzyme which can reduce 1 nmol of Cytochrome *c* per min.

Lauric acid hydroxylase activity was determined by Discovery Labware (BD Biosciences, Woburn, MA) using the method described by (Crespi et al. 2005). The reaction mixture (0.1 mL final volume) contained 20 µL yeast microsomal protein (1.1-4.0 mg/mL) expressing *C. gibbosum* P450s, 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 3.3 mM $MgCl_2$, 0.1 mM [14C]-lauric acid in 100 mM Tris buffer (pH 7.5). Human liver microsomes (HLM) (1.0 mg/mL) were used as a positive control. Yeast microsomes were incubated for 80 min at 25°C and HLM were incubated for 10 min at 37°C. Negative control incubations consisting of the same reaction mixture without microsomal proteins were also performed. All samples were run in single determinations. The reactions were stopped by the addition of 50 µL of 94% acetonitrile/6% acetic acid, centrifuged at 10,000 x g for 3 min, and 75 µL of supernatant was injected onto a C18 HPLC column (5 µ, 4.6 mm x 250 mm). The metabolites were separated at 45°C with methanol/acetonitrile/water (23:23:54) containing 1 mM perchloric acid adjusted to 100% methanol over 35 min with

a flow rate of 1.0 mL/min. Lauric acid and the ω-hydroxylated metabolite were detected by liquid scintillation counting.

Leukotriene B4 (LTB_4) hydroxylase activity was determined by Discovery Labware (BD Biosciences). The conversion of LTB_4 to its hydroxylated metabolites by recombinant CYP4 proteins and *Cyphoma* microsomes was performed by incubating 1.0 mg/mL of snail microsomes or 2.0 mg/mL of yeast microsomes with 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride, 29.7 μM LTB_4 in 100 mM KPO_4 (0.1 mL final volume). Human liver microsomes (0.5 mg/mL) were used as a positive control. Snail microsomes were incubated for 60 mins at 30°C, and yeast microsomes were incubated for 120 mins at both 23°C and 30°C. Reactions were terminated after 120 mins by the addition of 25 μL of 94% acetonitrile/6% glacial acetic acid. Incubations were centrifuged at 10,000 x g for 3 min. and 80 μL of the supernatant was injected onto a C18 HPLC column (5μ, 4.6 mm x 250 mm) and separated with an initial mobile phase consisting of 30% acetonitrile with 1 mM perchloric acid in water changing to 70% methanol over 20 minutes at a flow rate of 1.0 mL/min. Products were detected by monitoring absorbance at 270 nm and compared to known standards. Control reactions without microsomal protein, without NADPH and without incubation were performed with a 'global control' sample made by separately pooling all of the yeast microsomal samples and the snail microsomal samples.

Results

Identification of Cyphoma CYP4 genes

When an RT-PCR cloning approach was used with pooled digestive gland mRNA samples and degenerate primers designed to the conserved I-helix and heme-binding region, three partial CYP4 cDNAs approximately 396 base pairs (bp) in length were identified. The resultant sequences, which shared 41-54% amino acid identify, were separated into three clusters designated CYP4-1, CYP4-2, and CYP4-3. Specific primers designed to each of the three CYP4 clusters were used in 5' and 3' RACE reactions to obtain full-length sequences. In total, RACE and RT-PCR cloning efforts generated 352 cDNA clones. The cDNA clones within each cluster were aligned using ClustalX and used to construct three maximum parsimony trees to assist in the further identification of groups and subgroups of sequences within each cluster. These analyses were used to help infer the number of possible distinct CYP4 genes.

Based on current P450 Nomenclature rules, proteins that share $\geq$40% identity are assigned to the same family; proteins sharing $\geq$55% identity are grouped within the same subfamily; and allelic variants of the same gene are classified as having $\leq$ 3% divergence, unless functional differences (catalytic activities) can be demonstrated (Nelson 2006). The criteria established for P450 nomenclature, based on somewhat arbitrary cut-off values, does not take into account the phylogenetic relationships among species, whereby P450s sharing the same enzymatic function may be assigned to separate subfamilies because they fall below the arbitrary cut-off value (Nelson et al. 2004). Additionally, there are examples of distinct genes sharing greater than 97% amino acid identitiy, further complicating allelic variant vs. distinct loci assignment when genomic

information is lacking (Nebert et al. 1989). These caveats were considered here when classifying the CYP4 sequences identified from *Cyphoma*. Sequence analysis revealed fifteen full-length CYP4 cDNAs encoding twelve distinct CYP4 proteins, (CYP4-1, CYP4-2a, CYP4-2b, CYP4-3a1, CYP4-3a2, CYP4-3b1, CYP4-3b2, CYP4-3b3_clone 1, CYP4-3b3_clone 2, CYP4-3b3_clone 3, CYP4-3b3_clone 4, CYP4-3b3_clone 5, and CYP4-3c). ClustalX alignments indicate that the *Cyphoma* CYP4 protein sequences share identities ranging from 26.3% (CYP4-3a1v2 - CYP4-1v2) to 99.2% (CYP4-3b2_clone4 – CYP4-3b3/CYP4-3b2_clone5) (Figure 5).

CYP4-1 cluster. Maximum parsimony analysis (Figure 6) identified one CYP4 gene with several allelic variants. Upon further analysis two distinct groupings of variants were identified designated CYP4-1v1 and CYP4-1v2, which share 98.8% amino acid identity. CYP4-1v2 differ by a 15 bp insertion at 1201 bp position, resulting in an open reading frame of 1551 bp encoding a 517 amino acid protein rather than the 512 a.a. protein as seen in CYP4-1v1 (Figure 7). In addition, five of the 65 clones within the CYP4-1 group contained a 68 bp deletion at nucleotide position 1066 causing a frame shift to occur resulting in a premature stop codon. These clones likely represent a low frequency nonfunctional allele because this mutation would delete a signification portion of the CYP4, including the conserved cysteine residue in the P450 active site.

CYP4-2 cluster. This grouping is likely represented by two distinct genes, which share 93.5% amino acid identity and are designated here as CYP4-2a and CYP4-2b. The CYP4-2a subgroup is represented by only four of 121 possible clones, yet these sequences clearly form a distinct group as visualized by the unrooted maximum parsimony cluster analysis (Figure 8). The remaining clones grouped within the CYP4-

2b cluster likely represent two allelic variants within this subgroup, evidenced by a minor branch extending from the main CYP4-2b cluster in Figure 8. The nucleotide sequences of CYP4-2a and CYP4-2b are 1527 and 1545 bp, respectively, encoding proteins of 508 and 514 amino acids in length (Figure 9). The difference in length between the two cDNAs is due to an 18 bp addition to the 3' end of CYP4-2b. Both the CYP4-2a and CYP4-2b sequences contain this additional sequence; however, in the CYP4-2a group, a mutation at position 1527 leads to stop codon, truncating the protein six amino acids upstream of CYP4-2b stop codon. The highly similar UTRs suggest that CYP4-2a and CYP4-2b arose from a recent duplication event, followed by a diversification of both forms; however, which sequence is representative of the ancestral form is not known.

CYP4-3 cluster. The CYP4-3 cluster (the most diverse) includes 166 clones that may correspond to as many as nine distinct loci. An alignment of deduced amino acid sequences indicates that these group members share sequence identities ranging from 89.2% to 99.2% (Figure 5). Relationships among members of the CYP4-3 cluster were visualized by a maximum parsimony cluster analysis (Figure 10), indicating three separate groups (CYP4-3a, -3b, -3c) within the CYP4-3 members. The CYP4-3a group is likely represented by one gene with at least three allelic variants (CYP4-3a1v1, CYP4-3a1v2, and CYP4-3a2) which share >99% amino acid identity when positions common to all three variants are compared. The full-length CYP4-3a1v1, CYP4-3a1v2 and CYP4-3a2 sequences are 1563, 1500 and 1602 bp in length encoding proteins of 520, 499 and 533 amino acids, respectively (Figure 11, 12). One variant in particular designated CYP4-3a1v2, contains an inframe deletion of 63 bp (21 a.a.) causing the entire F helix and the majority of the SRS-2 region to be removed, resulting in a protein of 499 amino

acids. With the exception of this deletion, the protein coding sequences of CYP4-3a1v1 and CYP4-3a1v2 are identical. CYP4-3a2, represented by two clones, is 97.3% and 93.4% similar at the protein level to CYP4-3a1v1 and CYP4-3a1v2; however, if you disregard positions containing insertions/deletions, the similarity increases to 99.7% between all three variants. The most obvious difference between CYP4-3a1 and CYP4-3a2 sequences is the addition of 13 residues at the C-terminal end of CYP4-3a2, resulting from a single nucleotide substitution in the corresponding stop codon in CYP4-3a1.

Amino acid sequence identities between CYP4-3b members range from 95.0% to 99.2% and all encode proteins of 530 amino acids in length (Figures 11, 12). The CYP4-3b sequences can be further subdivided into one of three additional subgroupings (CYP4-3b1, -3b2, -3b3) supported by the maximum parsimony analysis (Figure 10). Protein identities range from 95.0 to 99.2% between CYP4-3b members (Figure 5). While consensus nucleotide sequences could be easily generated for CYP4-3b1 and CYP4-3b3 members so that at least one clone matched the consensus nucleotide sequence, this proved impossible for sequences within the CYP4-3b2 subgroup. Due to the extensive variation among CYP4-3b2 members, illustrated by the lack of clustering among clones in the MP analysis (Figure 10, Part B), five full-length clones (CYP4-3b2_clone 1 thru 5) were selected to represent the range of CYP4-3b2 diversity in lieu of generating a consensus nucleotide sequence with no physical representatives. Protein identities among the CYP4-3b2 subgroup members range between 96.1 to 99.2%. The greater than 97% amino acid identity among some subgroup members would suggest multiple allelic variants are a possibility.

The full-length CYP4-3c sequence is 1563 bp in length and encodes a protein of 520 amino acids. CYP4-3c sequences represent members that appear to be a hybrid[1] between CYP4-3a and CYP4-3b groups (Figures 11, 12), with amino acid identities ranging from 94.6% to 98.0% (CYP4-3c and CYP4-3a) and 94.2% to 96.9% (CYP4-3c and CYP4-3b). Percent identities increase even further if you restrict the region of comparison to the N-terminal half of CYP4-3c which is nearly identical to that of CYP4-3a, whereas the same can be said of the C-terminal half of CYP4-3c with CYP4-3b3.

A list of putative *Cyphoma* CYP4 genes, described here with their corresponding names assigned by the P450 Nomenclature Committee, can be found in Table 5. An amino acid alignment of the 15 full-length CYP4 cDNAs generated from nucleotide consensus sequences is shown in Figure 12.

Sequence analysis

BlastX searches with full-length cDNA sequences and phylogenetic analysis using both Bayesian and Maximum Likelihood methods (Figures 13, 14) confirmed that all of the *Cyphoma* sequences identified here belong to the cytochrome P450 4 clan and should be placed within the CYP4 family of sequences (Nelson et al. 2004). The CYP4 family is one of the oldest, most diverse families within the P450 superfamily (Simpson 1997). Because of this ancient lineage, the addition of new members in this family has blurred the definition of what constitutes a CYP family leading to 'family creep' or the inclusion of invertebrate members with less than 40% identity due to the reluctance to

[1] The term hybrid refers to a cDNA sharing identical blocks of sequence with two or more distinct cDNAs and does not imply a specific mechanism used to generate the hybrid cDNA

create new CYP families and the recognition that phylogenetic relationships need to be considered (Nelson et al. 2004).

Serial duplication events appear to have occurred within both the CYP4-2 (CYP4BK) and CYP4-3 (CYP4BL) lineages. The paralogous CYP4-2 and CYP4-3 *Cyphoma* sequences share less than 40% amino acid sequence identity with full-length vertebrate CYP4 members, and cluster within an apparently invertebrate-specific clade whose most closely related vertebrate homologs are found within CYP4 subfamilies A, B, F, T, X, Z (Figure 13). Top scores from BlastX searches indicate that *Cyphoma* CYP4-2 and CYP4-3 cDNAs are more similar to mammalian CYP4F members than to other non-mammalian CYP4F members or other vertebrate CYP4 subfamilies, suggesting possible convergent evolution between *Cyphoma* CYP4s and mammalian CYP4F forms, or the retention of residues reflecting a more ancestral form and indicating possible shared substrates between these two groups. A comparison of *Cyphoma* CYP4-2a, CYP4-2b to vertebrate CYP4 members found that *Cyphoma* cDNAs were most similar to mouse CYP4F13 (GenBank Accession # NP_570952.1) with amino acid identity ranging from 37.8 to 38.0%, compared to 32.9 to 33.0% with *Dicentrarchus labrax* CYP4F7 (seabass, GenBank Accession # AAD32564). Identities between *Cyphoma* CYP4-3 sequences and mouse CYP4F39 (GenBank Accession # AA145756.1) ranged from 36.4 to 37.3%, compared to 30.9 to 33.0% with *D. labrax* CYP4F7.

Cyphoma CYP4-1 (CYP4V10) sequences, however, do share >40% protein identity with other vertebrate CYP4V amino acid sequences, including 47.5-47.9% with zebrafish CYP4V2 (GenBank Accession # AAI25969) and 45.5-45.9% with human CYP4V2 (GenBank Accession # Q6ZWL3). *Cyphoma* CYP4-1 sequences also share

>40% identity with invertebrate sequences formally designated within the CYP4C subfamily that have recently been moved into the CYP4V subfamily and include 45.2-45.6% with *Carcinus maenas* CYP4V16 formally CYP4C39 (green crab, GenBank Accession # JC8026), and 41.3-41.7% with *Orconectes limosus* CYP4V11 - formally CYP4C15 (crayfish, GenBank Accession # AAF09264).

Several conserved domains are present within all of the *Cyphoma* CYP4 proteins. Beginning at the N-terminal portion, eukaryotic CYPs carry a sequence of amino acids responsible for targeting the P450 protein to the correct subcellular location in the membrane, which is absent in soluble bacterial P450 proteins (Figure 12). The highly conserved WXXXR domain towards the N-terminus is located in the center of the C-helix and neutralizes the charge of one of the propionate side chains of the heme group by the tryptophan nitrogen and the basic arginine (Graham & Peterson 2002). The I-helix, or P450 fingerprint region, is one of the most highly conserved domains among P450s. All of the *Cyphoma* CYP4 sequences contain the conserved threonine residue, located within the I helix, which is thought to aid in the protonation of the reduced oxygen intermediate (Williams et al. 2000a). The conserved EXXR domain found within the K-helix and located on the proximal side of the heme, may assist in stabilizing the heme core. Located just before the L-helix is the P450 consensus sequence (FXXGXRXCXG) containing the absolutely conserved cysteine serving as the fifth ligand to the heme iron (Werck-Reichhart & Feyereisen 2000).

Quantitative RT-PCR analysis of CYP4 expression

Quantitative RT-PCR analysis was used to investigate the constitutive and inducible expression of CYP4 forms in *Cyphoma* after dietary exposure to several gorgonian species with varying allelochemical profiles. Adult *Cyphoma* were allowed to feed ad libitum for 4 d on one of seven gorgonian diets or a control diet devoid of gorgonian allelochemicals. CYP4 transcripts were measured by quantitative RT-PCR from mRNA extracted from the digestive glands of either control or allelochemically-exposed snails using general and specific gene primers[2]. Digestive gland expression of CYP4 transcripts was monitored using general-cyp4-1, general-cyp4-2, general-cyp4-3 oligonucleotide primers and two additional primer pairs specific for a subset (A and B) of CYP4-3 sequences (Table 3). Figure 15 maps the location of those CYP4-3 clones that would be detected by CYP4-$3_{(sub\ A)}$ and CYP4-$3_{(sub\ B)}$ primers. Control amplifications were performed with cDNAs to confirm the specificity of the primers (data not shown).

Initially, CYP4 expression analysis was performed using only general-cyp4-1, general-cyp4-2, and the subset-specific CYP4-$3_{(sub\ A)}$ and CYP4-$3_{(sub\ B)}$ quantitative PCR primers (Figure 16). Subsequently, it was determined that CYP4-$3_{(subA)}$ and CYP4-$3_{(sub\ B)}$ primers did not detect some transcripts and the expression analysis was repeated with general-cyp4-3 primers for control snails and snails consuming the gorgonian diet *P. homomalla* for which a significant induction of CYP4-3 gene subsets was seen (Figure 17).

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[2] Three sets of general primers were designed to each of the three *Cyphoma* CYP4 clusters (CYP4-1, CYP4-2, CYP4-3) and were used to detect the expression of all sequences within the cluster. Two sets of specific primers, designated CYP4-3(sub A) and CYP4-3(sub B), were designed to pick up selected subsets of sequences within the CYP4-3 cluster only.

Significant differences in CYP4 expression between control and gorgonian diets were found for three gorgonian species (MANOVA, *G. ventalina* $F_{4,33} = 18.99$, $p < 0.001$; *P. americana* $F_{4,32} = 6.653$, $p < 0.001$; *P. homomalla* $F_{5,30} = 7.479$, $p < 0.001$; Table 6). However, for two of the two gorgonian diets a significant Diet x Reef interaction was observed (MANOVA, *G. ventalina* $F_{16,101} = 6.392$, $p < 0.001$; *P. homomalla* $F_{15,66} = 2.429$, $p < 0.001$, Table 6), indicating that CYP4 expression in snails inhabiting particular reefs were of high influence. ANOVA analysis further revealed that the significant Diet x Reef interaction in snails feeding on *G. ventalina* and *P. homomalla* could be traced to significant differences in CYP4-$3_{(subA)}$ expression among reefs (Table 7). Two snails collected from N. Normans out of 13 feeding on *G. ventalina* and one snail collected from Shark Rock out of 11 feeding on *P. homomalla*, showed negligible expression of CYP4-$3_{(subA)}$ transcripts, accounting for the significance of the interaction term. Interestingly, all three of these snails did express transcripts detected by subset-specific CYP4-$3_{(subB)}$ primers at levels similar to other snails feeding on the same diet. In addition, when the entire complement of CYP4-3 transcript expression was measured in control and *P. homomalla* fed snails using general-cyp4-3 primers, the single snail from the Shark Rock collection feeding on *P. homomalla*, originally showing anomalous gene expression for CYP4-$3_{(sub\ A)}$, displayed induced CYP4-3 transcript expression compared to reef controls. This finding may indicate that sequences detected by CYP4-$3_{(subA)}$ primers represent alternative splice variants or alleles that may not be expressed within certain subpopulations of snails. Sequences that are detected by CYP4-$3_{(subA)}$ primers are contained within the CYP4-3b1/CYP4-3b3/CYP4-3c groups defined by maximum parsimony analysis (Figure 15), all of which contain clones that are highly homologous to

each other at the C-terminal end. However, CYP4-3b1, CYP4-3b3, and CYP4-3c share between 94.2 and 96.9% protein identity, which falls below the 97% cut-off suggesting they are distinct genes. It is possible that these distinct loci may be inducible by specific compounds rather than structurally similar classes of allelochemicals produced by *P. homomalla*, and natural variability in allelochemical content among gorgonian colonies may account for differences in transcript expression.

In light of this information, the data were reanalyzed for both gorgonian diets excluding those reefs containing snails with the anomalously low CYP4-$3_{(subA)}$ expression values. As expected, there were no significant differences in CYP4 gene expression among snails feeding on *G. ventalina* (data not shown), but gene expression was still significantly different in snails feeding on *P. homomalla* compared to controls (MANOVA, $F_{5,24} = 7.606$, $p < 0.001$, Table 6). There was significant induction (ANOVA, $p < 0.001$, Table 7) of CYP4-2 (2.7-fold), CYP4-3 (2.7-fold), and CYP4-$3_{(subA)}$ (5.1-fold) gene expression in snails fed *P. homomalla* compared to controls (Figure 17) and the significant differences among *P. homomalla*-fed snails were limited to diet effects only. In addition, significant differences in CYP4 expression in *P. americana*-fed snails (MANOVA) were found not to be significant in ANOVA comparisons after p-values were Bonferroni adjusted (Table 7).

CYP4 gene expression did differ significantly among time-zero group snails collected from different reefs (MANOVA, $F_{20,70} = 1.739$, $p = 0.047$, Table 8, Figure 18). However, CYP4 gene expression did not vary among snails from different reefs fed a control diet for 4 days (MANOVA, $F_{20,80} = 1.367$, $p = 0.164$, Table 8, Figure 18). These results demonstrate that gene expression is highly variable among snails collected from

different reefs, possibility reflecting the variability of gorgonian diets at each reef location. If one considers the time-zero group as a proxy for the natural variation of gene expression in individuals on different reefs, then four days feeding on a control diet is enough to allow gene expression to return to some constitutive level, no matter reef origin, once the allelochemical stimulus is removed. Therefore, these results support the use of control snail gene expression as the true baseline, representing the non-allelochemically induced state of CYP4 gene expression in *Cyphoma*.

Running in parallel to the four day feeding assays was a longer term (14d) feeding assay used to investigate CYP4 gene expression in individuals exposed to the same gorgonian host almost five times longer than would be expected based on observations of residence time in the field (Harvell & Suchanek 1987). The design of the long term feeding assay was the same as described above, except one raceway contained ten tanks, each holding a single snail feeding on either a control diet (n = 2 snails) or one of seven gorgonian diets (n = 7 snails) for 14 d. All nine snails were collected from the same reef (i.e., North Normans). The mean CYP4 expression of two snails feeding on the control diet for 14d was no different from snails feeding on control diets for 4d (Figure 19). While the CYP4 expression results in snails feeding on a gorgonian diet are limited to one observation per gorgonian species, snails feeding on *B. asbestinum, E. mammosa, G. ventalina, P. acerosa, P. americana,* and *P. elisabethae* displayed CYP4 expression patterns similar to that of the paired controls (data not shown). Based on the four day feeding assays we expected to see sustained induction of CYP4-2, CYP4-3 and CYP4-$3_{(subA)}$ genes in the individual feeding on *P. homomalla* for 14d; there was higher CYP4-$3_{(subA)}$ gene expression in this snail compared to the control individuals, but expression of

CYP4-2 and CYP4-3 was similar to that of controls (Figure 19). However, feeding records indicate that the individual feeding on *P. homomalla* ceased feeding six days prior to the end of the 14d, which may explain the lack of CYP4 induction in this individual.

Identification of putative substrate recognition sites

Allelochemically-responsive *Cyphoma* CYP4 protein sequences were compared with characterized vertebrate fatty acid hydroxylases to help define the possible function and range of substrates for *Cyphoma* CYP4 forms by comparing key conserved residues among both groups. *Cyphoma* CYP4 proteins were also aligned with the crystal structure coordinates of bacterial fatty acid monooxygenase CYP102 (CYPBM3) (Ravichandran et al. 1993) and CYP2 forms described in (Gotoh 1992) to better define putative SRS regions and highlight differences between active site and substrate binding residues among *Cyphoma* P450 forms.

The bacterial CYP102 was used as a template to align *Cyphoma* CYP4-2 and CYP4-3 forms. In addition, human CYP4F3, CYP4F11 and rabbit CYP4A4, CYP4A5, CYP4A6, CYP4A7 were included in the alignment to aid in comparing previously annotated active site residues between mammalian fatty acid hydroxylases and *Cyphoma* CYP4 proteins (Figure 20). Amino acid identity between bacterial CYP102 and *Cyphoma* CYP4-2 and CYP4-3 sequences ranged between 16.6-21.5%. The bacterial CYP102 was used in place of one of the many crystallized eukaryote P450 forms (e.g., rabbit CYP2C5) because the CYP102 alignment with *Cyphoma* CYP4 genes resulted in

the highest percent identity (data not shown). Therefore, the bacterial CYP102 backbone was used as the template to define putative SRSs within the *Cyphoma* CYP4 proteins.

Even though *Cyphoma* CYP4 sequences are highly similar to one another within the clusters defined here, variation among even one residue can have a profound effect on enzymatic function (Lindberg & Negishi 1989). An alignment of the deduced amino acid sequences of both CYP4-2a and CYP4-2b indicates that the sequence variations are limited to 27 out of a possible 508 positions, not including the additional 6 residues at the C-terminal end of CYP4-2b. Seven of the 27 residues fall within five of the six substrate recognition sites (SRSs) which are critical for defining substrate specificity and activity in both vertebrate and invertebrate CYPs (Gotoh 1992, Pan et al. 2004). A similar deduced amino acid alignment constructed for all sequences found in the CYP4-3 group, indicated that sequence variation was limited to 40 out of 520 positions, with 13 substitutions occurring within SRSs. If we narrow our comparison to just the seven sequences representing the highly similar CYP4-3b subgroup (95.0 to 99.2% a.a. identity), 32 out of 520 possible positions contain amino acid variations, 13 of which are located in one of the six SRS regions.

The great diversity of P450 reactions stems from the structural arrangement of the P450 protein, allowing for the discrimination and orientation of substrates in close proximity to the activated oxygen species located in the heme core. Rigorous homology modeling of human CYP4A11 with bacterial fatty acid hydroxylase CYP102 coupled with site-directed mutational analysis has highlighted key resides in the binding pocket and substrate access channel influencing substrate specificity and hydroxylation position (Chang & Loew 1999). Table 9 contains a listing of these residues from CYP102 and

CYP4A11 with the corresponding residues from the *Cyphoma* CYP4-2 and CYP4-3 group of sequences. The order of residues listed in the table is based on their proximity to the heme core (upper - region nearest to the surface of the protein; middle - region buried in the middle located at distance from the ferryl oxygen; lower - region containing residues with 7 Å from the ferryl oxygen and site of metabolic activity). Despite the low sequence similarity overall, half of the residues within the lower substrate channel are absolutely conserved between the bacterial, human, and *Cyphoma* proteins, including T260, F261, G265, T268, A328 from CYP102. Three of the four remaining residues near the heme core in CYP102 (L75, A264, and A328) are replaced by bulkier residues in human CYP4A11 (Y120, E321, V386), which control the entrance and depth of substrate penetration within the active site and favor terminal carbon hydroxylation by restricting only the ω methyl group to come within proximity to the ferryl oxygen (Chang & Loew 1999). *Cyphoma* CYP4-2 and CYP4-3 forms also contain the same bulky residues as CYP4A11 within their active sites (Table 9), suggesting terminal hydroxylation would be favored.

In addition, within the active site of CYP4A, CYP4B, and CYP4F forms, a glutamate residue (E321 in CYP4A11 in Table 9) binds to the heme positioning the fatty acid into a position favoring ω-hydroxylation (Stark et al. 2005). Mutating this position to an alanine results in the reduction of terminal carbon hydroxylation (ω:ω-1) from 20:1 to 5:1, or the complete elimination of ω-hydroxylation all together (Dierks et al. 1998a, Dierks et al. 1998b) and reduced fatty acid hydroxylation (Zheng et al. 2003). *Cyphoma* CYP4-2b contains the less bulky alanine at the position equivalent to E321 in CYP4A11,

which would predict reduced hydroxylase activity and a substrate orientation favoring non-terminal ω hydroxylation for this cDNA.

Modeling studies of mammalian CYP4A (Chang & Loew 1999, Loughran et al. 2000) indicate that residues falling outside of the prescribed SRSs regions (Gotoh 1992) still play critical roles in substrate binding and activity (Figure 20). Many of these residues correspond to polymorphic sites within *Cyphoma* CYP4-2 and CYP4-3 proteins. For example, residues 81 and 91, forward of the first SRS region in the *Cyphoma* CYP4-3 cluster, are highly polymorphic and correspond to positions located at the mouth of the substrate access channel in CYP4A7 (R88) and CYP102 (Y51), responsible for anchoring the terminal carboxylic group on the fatty acid to the protein (Loughran et al. 2000). In general, *Cyphoma* CYP4-2 and CYP4-3 proteins seem to contain a majority of residues with small side chains at these positions (alanine, glycine, valine), suggesting that the opening of the substrate access channel may be able to accommodate larger fatty acids.

Further work by (Loughran et al. 2000) identified two additional residues (Y204 and F252) in rabbit CYP4A4, one of which is located outside of putative *Cyphoma* SRS sites defined here, that influence PGE_1 hydroxylase activity (Figure 20). These residues have been replaced by H206 and S255 in CYP4A7, which shows substantially less turnover of PGE_1 in comparison to CYP4A4. *Cyphoma* CYP4-2 proteins share the same residues at both corresponding positions in CYP4A4 (Y200 and F248), highlighting their potential role as prostaglandin (i.e., PGE_1) hydroxylases. *Cyphoma* CYP4-3 proteins share either tyrosine (Y) or histidine (H) at the first position corresponding to Y206 in CYP4A4, and contain a tryptophan (W) at the second position, corresponding to a conservative substitution from a similar aromatic phenylalanine (F) in CYP4A4.

Homology models of CYP4F3 and CYP4F11 based on bacterial (CYP102) and eukaryotic P450 crystal structure coordinates agree, for the most part, with our putative SRS assignments made for *Cyphoma* CYP4s based on our alignment to CYP102 (Kalsotra et al. 2004) (Figure 20). The exception is the SRS-2 region, which is slightly offset in comparison to *Cyphoma* CYP4 SRS-2, possibly due to the highly gapped nature of the author's alignment resulting from the variability seen among P450s within the F-G loop region (Peterson & Graham-Lorence 1995). The positioning, length and polarity of the F-G loop is thought to dictate the accessibility of the substrate to the access channel (Peterson & Graham-Lorence 1995, Williams et al. 2000b). The F-G loop in *Cyphoma* CYP4-2 and CYP4-3 is predicted to be substantially larger, containing 19 additional residues within the G helix, in comparison to CYP102. Interestingly, almost the entire F-helix has been removed from sequence CYP4-3a1v2, likely altering the substrate profile for this variant. Additionally, the putative SRS-1, -2 sites suggested here are different from those defined by (Loughran et al. 2000) for CYP4A members. It is likely that *Cyphoma* allelochemically-responsive CYPs share properties of both vertebrate CYP4A/F groups because of their evolutionary history and probable shared function. To date, no crystal structure has been determined for any eukaryotic CYP4 protein, further complicating the definitive identification of key residues for this family of proteins.

In summary, a majority of the amino acid substitution patterns among *Cyphoma* CYP4-2 (15 out of 27) and CYP4-3 (22 of 40) sequences cluster within SRS or near (within 2 residues) positions identified as being involved in the positioning of the substrate within the access channel or the active site. Although SRS-1 and 2 sites may shift somewhat depending on the species and subfamily used for comparison, these

patterns suggest that amino acid replacement differences are targeted within domains affecting substrate recognition.

Results of enzymatic assays

P450 content and reductase activity. The P450 content of *Cyphoma* microsomal proteins and recombinant yeast microsomes containing selected CYP4 cDNAs (Figures 21, 22) was investigated by the method of (Omura & Sato 1964). The characteristic P450 spectral peak was not detected in *Cyphoma* digestive gland microsomal preparations nor was a peak at 420 nm indicating the presence of degraded protein. Cytochrome P450 was consistently detected in recombinant microsomes from yeast induced for 8 hrs on galactose-containing media (27.4 pmol P450 mg protein^{-1} – 118.7 pmol P450 mg protein^{-1}), but not in yeast induced for 15 hrs. No 420 nm peak was ever observed in any yeast microsomal preparations. The lack of chromophore detection at 450 nm with spectrophotometry in some yeast samples does not preclude NADPH-dependent enzymatic activities in these yeast microsomes. For example, recombinant yeast expression of ovine CYP4F21 yielded no P450 spectra, but yeast microsomes containing CYP4F21 metabolized three stable PGH_2 analogues and PGE_2 to their respective 20-hydroxy metabolites, whereas control microsomes lacked such activity (Bylund 2000, Bylund & Oliw 2001). NADPH-cytochrome P450 reductase activity was also confirmed to be strongly induced in galactose-induced yeast microsomal samples (~ 5000 nmol min^{-1} mg protein^{-1}) and highly expressed in the one snail digestive gland examined (631 nmol min^{-1} mg protein^{-1}).

Fatty acid hydroxylase activity. Yeast microsomes containing *Cyphoma* CYP4-2 and CYP4-3 (Figure 22) proteins were surveyed for both lauric acid and leukotriene B4 (LTB_4) hydoxylase activity to define a range of possible fatty acid substrates for *Cyphoma* CYP4 proteins. Recombinant yeast microsomes, isolated from 8- and 15-hr induced cultures expressing either CYP4-2a, CYP4-2b, CYP4-3a1, CYP4-3b1, or CYP4-3b3, were incubated with 0.1 mM lauric acid or 29.7 μM LTB_4 and the production of hydroxylated metabolites was analyzed by HPLC or liquid scintillation detection. Yeast microsomes containing recombinant CYP4 protein did not metabolize lauric acid to any detectable metabolites; however, leukotriene B4 hydroxylase activity was detected in recombinant yeast microsomes (Table 10, Figure 23). LTB_4 activity was present only in yeast expressing CYP4-3a1 and CYP4-3b3 ranging from 0.017 – 0.299 pmol mg^{-1} min^{-1} and was greater in samples incubated at 30°C then at 23°C. In addition, LTB_4 hydroxylase activity was detected in the digestive gland of three of the four snails feeding on *P. homomalla*, while only one of the ten snails feeding on the control diet exhibited detectable activity (Table 11). Human liver microsome samples were run in parallel experiments and showed typical activity and metabolic profile for this enzyme source.

Discussion

This study provides the first evidence linking the induction of specific P450 transcripts in a marine consumer to the chemistry of its gorgonian host. The diet of the generalist *C. gibbosum* includes a variety of gorgonian families with structurally diverse allelochemical profiles. Only snails feeding on the gorgonian *Plexaura homomalla*

displayed significant induction (2.7- to 5.1-fold) of digestive gland CYP4 transcripts in comparison to individuals feeding on a control diet devoid of gorgonian allelochemicals.

The suite of allelochemicals produced by *P. homomalla* is unique to this genus of Caribbean coral and includes high tissue concentrations of prostaglandins (1 to 8% of the dry tissue weight) (Weinheimer & Spraggins 1969, Schneider et al. 1977a, Dominguez et al. 1980). In mammalian systems, prostaglandins behave as 'local' (i.e., autocrine or paracrine) lipid mediators, acting through G-protein-coupled receptors to stimulate the pro-inflammatory cascade after tissue injury, sensitize neurons to pain, stimulate smooth muscle contraction, regulate vasodilation, regulate temperature, and control ionic balance in the kidney (Funk 2001, Rowley et al. 2005). In invertebrates, prostaglandins play similar roles as modulators of ion transport and regulators of immune response and reproduction (Stanley 2000, 2006), but are most notable as prey chemical defenses in marine systems (Gerhart 1984, Di Marzo et al. 1991, Gerhart 1991, Marin et al. 1991).

The most abundant prostaglandin in *P. homomalla* is the fully esterified PGA_2 form (averaging 1-2% of dry weight), followed by its hydroxy acid (0.2% of dry weight) (Weinheimer & Spraggins 1969, Schneider et al. 1977a, Dominguez et al. 1980). Damage to gorgonian soft tissue can stimulate the lipase-mediated enzymatic hydrolysis of the acetoxy methyl ester of PGA_2, resulting in the accumulation of the partially esterified PGA_2 compounds (Schneider et al. 1977a, Valmsen et al. 2001). Palatability studies with a natural assemblage of reef fish have confirmed the ichthyodetterency of the crude extract of *P. homomalla* (O'Neal & Pawlik 2002), the hydroxy acids of PGA_2 (both S and R isomers), and both the hydroxyl methyl ester and acetoxy acid of PGA_2 (Gerhart 1984, Pawlik & Fenical 1989). Interestingly, the fully-esterified form of PGA_2 did not

deter consumption; however, esterified prostaglandins have been hypothesized to function as sources of inducible defenses, whereby damage to the coral may cause the innocuous acetoxy methyl esters to undergo hydrolysis to the noxious hydroxy acids over a period of several hours (Pawlik & Fenical 1989). The esterification of prostaglandins may protect the gorgonian from self-intoxication, allowing for the high concentration of prostaglandin compounds to persist in gorgonian tissues. For less mobile predators, like *C. gibbosum*, feeding stimulated formation of the hydroxy acids of PGA_2 in *P. homomalla* hosts and/or acidic hydrolysis of esterified PGA_2 forms in the low-pH gastric environment could pose a significant threat to these obligate gorgonian predators, unless they possess orchestrated detoxification mechanisms capable of responding to their host allelochemicals.

Within the diverse P450 superfamily, family 4 monooxygenases (CYP4) contain the major fatty acid ω-hydroxylases capable of preventing lipotoxicity (Hsu et al. 2007). Phylogenetic analysis of *C. gibbosum* P450 sequences revealed that those transcripts induced in response to dietary exposure of *P. homomalla* are most closely related to vertebrate CYP4A and CYP4F forms well known for their ability to metabolize prostaglandins, including PGA_2 (Sawamura et al. 1993, Okita & Okita 2001, Capdevila & Falck 2002, Kikuta et al. 2002, Kalsotra & Strobel 2006). In some cases the snail CYP4s share the same amino acid residues determined to be important in prostaglandin metabolism by vertebrate CYP4A/F forms. Allelochemical-responsive P450s in *Cyphoma* include both CYP4-2 and CYP4-3, which likely function in a cooperative fashion to mediate prostaglandin detoxification. The induction of P450 forms by gorgonian diets did not extend to all *Cyphoma* CYP4 forms. CYP4-1 (CYP4V10) was

not induced, nor would this have been expected, since it falls within the CYP4C/V clade containing CYP forms whose roles have been postulated to include hormone-stimulated fatty acid oxidation during starvation (cockroach, CYP4C1) (Lu et al. 1996, 1999) and fatty acid metabolism in the retina in vertebrates (reviewed in Hsu et al. 2007).

P450 genes, in contrast to housekeeping genes, are often transcriptionally induced by substrates upon which they act (Whitlock 1986). A significant increase in mRNA for selected CYP4 transcripts was observed in snails following a 4 d dietary exposure to *P. homomalla* allelochemicals. Both CYP4-2 and CYP4-3 were constitutively expressed and induced to the same degree (2.7-fold). This combination of constitutive and inducible expression mirrors that of the CYP6B17 and related transcripts in *Papilio glaucus*, a generalist lepidopteran that feeds on a range of host plants and occasionally encounters toxic furanocoumarins in its diet (Li et al. 2001). CYP6B17 is constitutively expressed in uninduced larvae and induced 3-fold in larvae exposed to the furanocoumarin xanthotoxin. For generalist species like *P. glaucus* and *C. gibbosum*, where the chance of encountering an allelochemically-rich diet is high, there may be a selective advantage to constitutively expressing select P450 forms. *P. glaucus* also expresses a second group of furanocoumarin metabolizing genes that are closely related to the CYP6B17 group. These transcripts within the CYP6B4 group are not constitutively expressed in unexposed larvae, but are highly inducible upon exposure to furanocoumarins (Li et al. 2001). In contrast, all of the allelochemically-responsive genes had some measurable level of constitutive expression in control-fed snails; however, additional quantitative PCR analysis of a subset of *Cyphoma* CYP4-3 genes revealed CYP4-$3_{(\text{sub A})}$ transcripts exhibited a greater fold induction (5.1-fold) and lower

basal expression in comparison to the CYP4-3 cluster as a whole. This result suggests selective CYP4-3 forms have different patterns of inducibility, perhaps to respond to different suites of compounds produced by *P. homomalla*.

Since their initial discovery of prostaglandins in gorgonians by Weinheimer and Spraggins in 1969, the synthesis (Valmsen et al. 2001, Valmsen et al. 2004), structural diversity (Spraggins 1972, Schneider et al. 1977b, Agalias et al. 2000, Schneider et al. 2002, Schneider et al. 1972, Light & Samuelsson 1972, Schneider et al. 1977a, Groweiss & Fenical 1990) and bioactivity of these compounds (Honda et al. 1987, Bhakuni & Rawat 2005, Chiang et al. 2006, Roberts et al. 2003) have been studied intensively. Prostaglandins in the A series (i.e., PGA_2) dominate the bulk of the crude extract of *Plexaura homomalla* (Schneider et al. 1977a). The 15(R) form of PGA_2 is typically found in Florida Keys populations, whereas the typical mammalian 15(S) form is found in colonies collected in the Bahamas, Cayman Islands and Curaçao (Schneider et al. 1977b, Ciereszko et al. 1985, Gerhart 1986, Schneider et al. 2002). Generally, individual *P. homomalla* colonies produce either the R and S configurations, but mixtures within the same colony have been reported (Schneider & Morge 1971, Schneider et al. 1977a). Several PGA_2 analogues of minor abundance have also been described; these include 5,6-*trans*-PGA_2 (5-15% of the PGA_2 fraction) (Schneider et al. 1977a), and 13,14-dihydro- and 13,14-*cis*-PGA_2 derivatives (Schneider et al. 1977b). Bahamian *P. homomalla* populations have also been reported to contain the prostaglandin E_2 (PGE_2), methyl ester and 15-acetate methyl ester at roughly 10% of the abundance of PGA_2 esters (Schneider et al. 1972, Prince et al. 1973, Schneider et al. 2002) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) as both the acid and the acetate methyl ester (0.6% of the chloroform extract) (Groweiss &

Fenical 1990). It is possible that the structurally diverse pool of prostaglandins found in gorgonian tissues may have necessitated the evolution of multiple prostaglandin metabolizing P450s, resulting in the diversity of closely related CYP4-3 transcripts identified here. Many of the amino acid differences (22/40) among members of the CYP4-3 group occur within, or near, the putative six SRS regions or residues lining the substrate access channel. Based on the location of these differences and knowledge gained from site-directed mutagenesis studies, it is likely that these variations will confer differences in catalytic activity and substrate specificity among these closely related proteins.

It is conceivable that ancestral *Cyphoma* P450s, whose function was to metabolize endogenous fatty acids, acquired new functions through strategic mutations allowing this predator to exploit prostaglandin-rich prey. One recent study suggests that the loss of host plant specialization in the *Papilio* lineage, favoring generalists like *P. glaucus*, may have evolved from only a small number of mutational changes within the SRS-6 region, allowing for the acquisition of novel catalytic activities while still retaining the ancestral furanocoumarin-metabolizing capability (Mao et al. 2007). This gradual accumulation of functionally significant replacements, often following a series of gene duplications (Wen et al. 2006), has been hypothesized as a means for enzymes to evolve in response to host selection (Mao et al. 2007). It would be interesting to explore whether the co-occurring specialist *Cyphoma signatum*, which feeds exclusively on *Plexaurella* spp., known only to contain the eicosanoid 11-R-hydroxy-5Z,8Z,12E,14Z-eicosateraenoic acid (11-R-HETE) (Di Marzo et al. 1996) and not prostaglandins, retains a similar diversity CYP4 transcripts capable of responding to foreign dietary prostaglandins. Insect studies

indicate that the constitutive expression, inducibility and catalytic versatility of herbivore P450s generally correspond to the frequency of ecological allelochemical exposure (Cohen et al. 1992, Li et al. 2001, Li et al. 2007). This theory would predict that while *C. signatum* may share similar CYP4 genes, *C. gibbosum* would be more efficient at metabolizing dietary prostaglandins.

The expansion of the CYP4-3 cluster relative to the other *Cyphoma* P450s identified in the present study suggests positive selection of this subfamily likely through repeated gene duplication events. In addition, alternative splicing of transcripts or the formation of chimeric CYPs from the splicing of pre-mRNA molecules can allow for the generation of novel genes with divergent catalytic functions. There is evidence for both of these scenarios within the P450 superfamily. For example, the human CYP4F3 gene has two different inducer specific 5' UTR transcriptional start sites capable of controlling the mutually exclusive splicing of either exon 3 or 4, generating the tissue-specific expression of catalytically distinct CYP4F3A and CYP4F3B isoforms (Christmas et al. 1999, Christmas et al. 2001). In addition, chimeric RNA molecules have been identified for both CYP2C and CYP3A members (Finta & Zaphiropoulos 2000, Finta & Zaphiropoulos 2002). In this case, exons are joined together from distinct pre-mRNA molecules yielding chimeric mRNAs. Formation of hybrid mRNA, especially by trans-splicing, allows detritus or solo exons to become functional even if they are dispersed in the genome (Bonen 1993). In both cases, exon shuffling or alternative splicing of distinct transcripts provides the opportunity for novel catalytic functions to emerge, further increasing the diversity of P450 genes. However, without specific knowledge of the

genomic arrangement or exon-intron structure of *Cyphoma* CYP4 genes, we cannot distinguish between these and other possibilities.

The consensus amino acid sequence representing *Cyphoma* CYP4-3c (clones within this group share greater than 96.3% a.a. identity) is a hybrid of both the CYP4-3b and CYP4-3a groups, with individual clones spanning a range of identities between the 3a and 3b nodes (Figure 10). This suggests the CYP4-3c locus defined here could be made up of multiple loci resulting from repeated duplication events, one locus with highly polymorphic alleles, or products of alternative splicing or trans-splicing. The same could also be said of the CYP4-3b2 subgroup represented by five unique clones. CYP4-3b2_clone 5 is a hybrid between CYP4-3b2_clone 3/CYP4-3b2_clone 4/CYP4-3b3 (N-terminal) and CYP4-3a group (C-terminal). Alternatively, the hybrid nature of CYP4 forms here may have resulted from the combination of gene duplication events followed by gene conversions, resulting in the homogenization present among members of the CYP4-3 group. This scenario has been hypothesized to account for the nearly identical amino acid sequences in CYP6B gene paralogs in the moth, *Helicoverpa zea* (Li et al. 2002b). Additional studies such as genomic PCR or the construction of a BAC library would help precisely identify the number of distinct loci represented by the CYP4-3 cluster.

Multiple gene duplication events are common among CYP4 members, including human CYP4F genes (CYP4F2, CYP4F3(A/B), CYP4F8, CYP4F11, CYP4F12) (Nelson et al. 2004). While these genes share greater than 77% amino acid identity with each other, their enzymatic activities and substrate specificities can be strikingly different (reviewed by Kalsotra & Strobel 2006). A closer examination of the substrate spectrum

for vertebrate fatty acid hydroxylases suggests certain isoforms have narrow substrate specificity, which is a significant departure from those xenobiotic CYP families (1, 2, 3) known for their broad substrate pool. Consequently, the lack of lauric acid hydroxylase activity or substantial LTB_4 hydroxylase activity in recombinant yeast microsomes reported here does not preclude fatty acid hydroxylase activity of *Cyphoma* CYP4 proteins. For example, recombinant rabbit CYP4A4 can metabolize long chain fatty acids, including prostaglandins, yet shows little if any ability to metabolize lauric acid (reviewed in Okita & Okita 2001). Mouse CYP4F14 catalyzes the ω-hydroxylations of LTB_4, lipoxin A_4 and PGA_1, but no activity towards lipoxin B_4, lauric acid or arachidonic acid was detected (Kikuta et al. 2000). Similarly, not all LTB_4 ω-hydroxylases catalyze prostaglandin hydroxylation. For example, human CYP4F3 shares LTB_4 ω-hydroxylase activity with rodent CYP4F14, yet it does not catalyze the hydroxylation of PGA_1, PGE_1, or lauric acid (Kikuta et al. 1998). It is possible that the substrate specificities of allelochemically-responsive *Cyphoma* CYP4 forms might be finely tuned toward those fatty acids (primarily prostaglandins) found within its gorgonian diet. Even so, LTB_4 hydroxylase activity measurements in *Cyphoma* microsomes suggests those snails exposed to prostaglandin-rich gorgonian diets have a greater ability to metabolize fatty acids in comparison to snails feeding on control diets, and it is likely that this enhanced enzymatic activity is due to the induction of allelochemically-responsive CYP4s. *In vitro* studies will be needed in order to directly demonstrate that ecologically relevant prostaglandins can serve as substrates for *C. gibbosum* P450s; nevertheless, this study provides the most compelling evidence thus far for the involvement of P450s in allelochemical metabolism in marine consumers.

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Table 1. Summary of digestive gland RNA and protein samples collected during January 2006 feeding assays.

	Big Point		North Normans		Rainbow Gardens		Shark Rock		Sugar Blue Holes		
Diet/group	RNA	Protein	RNA	Protein	RNA	Protein	RNA	Protein	RNA	Protein	**Total**
B. asbestinum	2	1	2	0	5	1	2	1	2	1	17
E. mammosa	2	1	2	0	4	1	2	1	2	1	16
G. ventalina	2	1	2	0	5	1	2	1	2	1	17
P. acerosa	2	1	2	0	2	1	2	1	2	1	14
P. americana	2	1	2	0	3	2	3	2	2	2	19
P. elisabethae	2	0	2	0	0	0	0	0	2	0	6
P. homomalla	2	1	2	0	3	1	2	1	2	1	15
Control	6	3	6	0	9	3	6	3	6	3	45
Time Zero	6		6		6		6		7		31
Total	26	9	26	0	37	10	25	10	27	10	180

Numbers indicate the quantity of digestive glands processed in each category.
This table does not include snails that perished during the feeding assays or digestive gland RNA samples that were damaged during isolation and rendered unusable.

Table 2. Degenerate and specific oligonucleotide primers used in the present study

Gene(s)	Primers	Direction	Sequences (5' to 3')	Application
CYP4	CYP4_F1	Forward	GAY ACI TTY ATG TTY GAR GG	Amplification of partial cDNA
	CYP4_F2	Forward	ATG TTY GAR GGI CAY GAY ACI AC	
	CYP4_R3	Reverse	GCR ATY TTY TGI CCI ATR CAT TT	
Adaptor primers	RACE_1_F		AAT ACG ACT CAC TAT AGG	Adaptor Primers for RACE
	AP1		CCA TCC TAA TAC GAC TCA CTA TAG GGC	
CYP4-1	CYP4_F11_R	Reverse	TGA CGT GAA GAC TAT CAC TGT GAC	5' RACE
CYP4-2	CYP4_A12_R	Reverse	GAA GAT ATG GAT CGT ACA TAC CG	5' RACE
	CYP4-2_F1	Forward	GAG AAG TTT TCA GCA ACA TGG ACT TGG	Full-length cloning of CYP4-2 group
	CYP4-2_R1	Reverse	GGA AGA TCA CCG TGT GTG AAA GGA C	
CYP4-3	CYP4_D09_F	Forward	ACC TTG TAC AGC CTG GCG AGA TGG	3' RACE
	CYP4_D09_R	Reverse	CCA AAT ATC TAT TGC TAT GAA TG	5' RACE
	CYP4-3_R1	Reverse	AGT GGC CAA TGG AGC ACG TAG G	5' RACE
	CYP4-3_R2	Reverse	GCA GGT AGT CAT GTG GCC TGT C	5' RACE
	CYP4-3_F3	Forward	ACA AGA CGT TTC GAC AGA ACA GTT AGC	Full-length cloning of CYP4-3 group
	CYP4-3_R6	Reverse	TTG CTG TCC CAC AGT GTC AAC AAG	
Actin	DegAct5	Forward	ACA ACG GYT CSG GYA TGT GC	Degenerate actin primers
	DegAct3	Reverse	GAA GCA YTT GCG RTG WAC RAT	

Table 3. Quantitative PCR primers used in the present study

Gene target[a]	Primers	Direction	Sequence (5' to 3')
CYP4-1	F11_58_23_ext	Forward	GCT GAC TCC AAC TTT CCA CT
	R3_58_23_ext	Reverse	GGC CCT TAT CTA CAT GAT GC
CYP4-2	F6_60_1_ext	Forward	GGG AGA TCT GCA CAA GCT CC
	R15_60_1_ext	Reverse	GAA AGG GCC GAG CCA AAA GC
CYP4-3$_{(sub\ A)}$	F12_54_9_ext	Forward	ATG CCC TGT TAC AGG GAC GT
	R11_54_9_ext	Reverse	GTC CGT CCA GGT TCA AAG GT
CYP4-3$_{(sub\ B)}$	F1_54_4_ext	Forward	TGA TCT GAC TCA GCT GCC ATA C
	R1_54_4_ext	Reverse	CTC TGT GAT TTC TCG CTC AAT G
CYP4-3	197_all_F1	Forward	TTT GCC ATG AAT GAA TTG AAG GTG
	197_all_R1	Reverse	GGA GCA CGT AGG ATT TCA TGA TTT GG
Actin	F3_CgActin	Forward	TCG GTC GTC CCA GAC ATC AGG
	R1_actinRT	Reverse	TCT CCA TGT CGT CCC AGT TGG TG

[a] CYP4-3$_{(sub\ A)}$ and CYP4-3$_{(sub\ B)}$ detect select sequences within the CYP4-3 cluster (see Figure 15). CYP4-3$_{(sub\ A)}$ detects clones within the following groups/subgroupings: CYP4-3b1, CYP4-3b3, CYP4-3c. CYP4-3$_{(sub\ B)}$ detects clones within the following groups/subgroupings: CYP4-3a, CYP4-3b2, CYP4-3c.

Table 4. Oligonucleotide primers used for recombinant *C. gibbosum* CYP4 expression in yeast

Gene	Clone[a]	Primers	Direction	Sequence (5' to 3')
CYP4-2	198_27	pENTR_198_27_F1	Forward	CAC CAT GGA CTT GGG TTT TTC CTC C
		pENTR_198_27_R1	Reverse	ATG TTC CCA GTT GTT ATA TAC AGT GGG TGT
	198_58	pENTR_198_27_F1	Forward	CAC CAT GGA CTT GGG TTT TTC CTC C
		pENTR_198_58_R1	Reverse	TAC AGT GGG TGT TCT TGG AGT GGC AAA CAT
CYP4-3	197_48/ 197_52/ 197_53	pENTR_197_53_F1	Forward	CAC CAT GGA TGA TAC CTT CTC TCA ACT G
		pENTR_197_53_R1	Reverse	GCC TTT GCG TGG CGT GAT

[a] Clone 198_58 is representative of CYP4-2a; clone 198_27 is representative of CYP4-2b; clone 197_48 is representative of CYP4-3b1; clone 197_52 is representative of CYP4-3a1; clone 197_53 is representative of CYP4-3b3.

Table 5. Summary of *C. gibbosum* cytochrome P450 nomenclature

Cluster	Genes	Nomenclature[a]	Named alleles[b]	GenBank Accession #
CYP4-1	CYP4-1v1	CYP4V10	CYP4V10v1	EU546250
	CYP4-1v2		CYP4V10v2	EU546251
CYP4-2	CYP4-2a	CYP4BK1		EU546252
	CYP4-2b	CYP4BK2		EU546253
CYP4-3	CYP4-3a1v1	CYP4BL1	CYP4BL1v1	EU546254
	CYP4-3a1v2		CYP4BL1v2	EU546255
	CYP4-3a2		CYP4BL1v3	EU546256
	CYP4-3b1	CYP4BL4		EU546257
	CYP4-3b2_clone1	CYP4BL5		EU546258
	CYP4-3b2_clone2	CYP4BL6		EU546259
	CYP4-3b2_clone3	CYP4BL7		EU546260
	CYP4-3b2_clone4	CYP4BL8		EU546261
	CYP4-3b2_clone5	CYP4BL9		EU546262
	CYP4-3b3	CYP4BL3		EU546263
	CYP4-3c	CYP4BL2		EU546264

[a] Official names assigned by the P450 Nomenclature committee. Sequence analysis suggests twelve distinct CYP4 loci exist in *C. gibbosum*.
[b] Note: not all possible alleles are shown or have been named by the P450 Nomenclature committee.

Table 6. Results of a two-way MANOVA investigating differences in digestive gland CYP4 gene expression in *C. gibbosum* feeding on control versus gorgonian diets.

Factors	df	Wilks' Λ	*F*	p
Control vs. *B. asbestinum*				
Diet	4, 33	0.933	0.590	0.672
Reef	16, 101	0.536	1.435	0.140
Diet x Reef	16, 101	0.744	0.644	0.840
Control vs. *E. mammosa*				
Diet	4, 33	0.887	1.015	0.414
Reef	16, 98	0.598	1.127	0.341
Diet x Reef	16, 98	0.594	1.142	0.329
Control vs. *G. ventalina*				
Diet	4, 33	0.303	18.99	**<0.001**
Reef	16, 101	0.116	6.476	**<0.001**
Diet x Reef	16, 101	0.118	6.392	**<0.001**
Control vs. *P. acerosa*				
Diet	4, 30	0.816	1.682	0.180
Reef	16, 92	0.470	1.615	0.080
Diet x Reef	16, 92	0.580	1.126	0.344
Control vs. *P. americana*				
Diet	4, 32	0.546	6.653	**<0.001**
Reef	16, 98	0.574	1.227	0.261
Diet x Reef	16, 98	0.586	1.175	0.301
Control vs. *P. elisabethae*				
Diet	4, 15	0.480	4.067	0.019
Reef	8, 30	0.381	2.328	0.045
Diet x Reef	8, 30	0.619	1.017	0.444
Control vs. *P. homomalla* (All reefs included)				
Diet	5, 30	0.445	7.479	**<0.001**
Reef	20, 100	0.279	2.355	**<0.001**
Diet x Reef	20, 100	0.270	2.429	**<0.001**
Control vs. *P. homomalla* (deletion of Shark Rock individuals)				
Diet	5, 24	0.387	7.606	**<0.001**
Reef	15, 66	0.439	1.542	0.115
Diet x Reef	15, 66	0.421	1.632	0.089

A $p \leq 0.002$ should be accepted as significant (Bonferroni adjusted). Significant values in bold.

Table 7. Results of ANOVA comparisons (Univariate F-tests) of diet- and reef-specific mean CYP4 gene expression in *C. gibbosum* feeding on control vs. gorgonian diets.

Diet comparisons		df	MS	F	p
Control vs. *G. ventalina*					
Diet	CYP4-1	1, 36	0.016	0.050	0.825
	CYP4-2	1, 36	0.030	0.550	0.464
	CYP4-3$_{(subA)}$	1, 36	6.070	46.339	**<0.001**
	CYP4-3$_{(subB)}$	1, 36	0.010	1.368	0.250
Reef	CYP4-1	4, 36	0.069	0.211	0.931
	CYP4-2	4, 36	0.054	0.989	0.426
	CYP4-3$_{(subA)}$	4, 36	3.127	23.869	**<0.001**
	CYP4-3$_{(subB)}$	4, 36	0.124	1.710	0.169
Diet x Reef	CYP4-1	4, 36	0.280	0.850	0.503
	CYP4-2	4, 36	0.034	0.629	0.645
	CYP4-3$_{(subA)}$	4, 36	3.555	27.137	**<0.001**
	CYP4-3$_{(subB)}$	4, 36	0.032	0.447	0.774
Control vs. *P. americana*					
Diet	CYP4-1	1, 35	0.037	0.114	0.738
	CYP4-2	1, 35	0.035	0.871	0.357
	CYP4-3$_{(subA)}$	1, 35	0.948	6.681	0.014
	CYP4-3$_{(subB)}$	1, 35	0.354	5.667	0.023
Control vs. *P. homomalla* (All reefs included)					
Diet	CYP4-1	1, 34	0.162	0.442	0.511
	CYP4-2	1, 34	1.557	36.592	**<0.001**
	CYP4-3$_{(subA)}$	1, 34	0.394	1.316	0.259
	CYP4-3$_{(subB)}$	1, 34	0.494	8.210	0.007
	CYP4-3	1, 34	1.678	19.924	**<0.001**
Reef	CYP4-1	4, 34	0.643	1.756	0.161
	CYP4-2	4, 34	0.043	1.017	0.412
	CYP4-3$_{(subA)}$	4, 34	3.200	10.698	**<0.001**
	CYP4-3$_{(subB)}$	4, 34	0.041	0.688	0.605
	CYP4-3	4, 34	0.078	0.925	0.461
Diet x Reef	CYP4-1	4, 34	0.053	0.144	0.965
	CYP4-2	4, 34	0.090	2.113	0.101
	CYP4-3$_{(subA)}$	4, 34	2.354	7.870	**<0.001**
	CYP4-3$_{(subB)}$	4, 34	0.036	0.592	0.670
	CYP4-3	4, 34	0.144	1.713	0.170
Control vs. *P. homomalla* (deletion of Shark Rock individuals)					
Diet	CYP4-1	1, 28	0.097	0.488	0.490
	CYP4-2	1, 28	1.530	32.658	**<0.001**
	CYP4-3$_{(subA)}$	1, 28	3.877	27.577	**<0.001**
	CYP4-3$_{(subB)}$	1, 28	0.509	7.610	0.010
	CYP4-3	1, 28	1.765	18.754	**<0.001**

A $p \leq 0.007$ should be accepted as significant (Bonferroni adjusted). Significant values in bold.

Table 8. Results of a one-way MANOVA investigating CYP4 gene expression variability among reefs for time-zero and control-fed *C. gibbosum*. Reefs were considered random factors.

Factors	df	Wilks' Λ	*F*	p
Reef (Experiment 1)				
Control diet	20, 80	0.163	1.367	0.164
Reef (Experiment 2)				
Time-zero group	20, 70	0.265	1.739	**0.047**

Table 9. Comparison of amino acid residues lining the substrate binding channel in CYP102(BM3), CYP4A11 and *C. gibbosum* CYP4-2 and CYP4-3 proteins.

Location	Domain	SRS	CYP102(BM3)	4A11	4-2a	4-2b	4-3a1v1	4-3a2	4-3a1v2	4-3c	4-3b2_clone3	4-3b2_clone4	4-3b3	4-3b2_clone5	4-3b2_clone2	4-3b1	4-3b2_clone1
Upper	near A		L20	L66	M63	M63	P60	P60	P60	P60	P60	P60	P60	P60	P60	P60	P60
Upper	A		P25	E71	R68	R68	G64	G64	G64	G64	G64	G64	G64	G64	G64	G64	G64
Upper	A		L29	I75	F72	**Y72**	**D68**	**D68**	**D68**	**D68**	Q68	Q68	Q68	Q68	Q68	Q68	Q68
Upper	β1		R47	K94	Q91	**H91**	R87	R87	R87	R87	R87	R87	R87	R87	R87	R87	R87
Upper	β2		Y51	R96	V95	V95	**I91**	**I91**	**I91**	**I91**	V91	V91	V91	V91	**A91**	**A91**	**A91**
Upper	B'	SRS-1	S72	H117	G115	**N115**	S111	S111	S111	S111	S111	S111	S111	S111	**N111**	**N111**	**N111**
Upper	F	SRS-2	L188	V232	I228	I228	S230	S230	--	S230	S230	S230	S230	S230	S230	S230	S230
Middle	A		V26	L72	I69	I69	I65	I65	I65	I65	I65	I65	I65	I65	I65	I65	I65
Middle	B'	SRS-1	A74	S119	--	--	V117	V117	V117	V117	V117	V117	V117	V117	**G117**	**G117**	**G117**
Middle	K/β5	SRS-5	P329	P387	P381	P381	P395	P395	P374	P395	P395	P395	P395	P395	P395	P395	P395
Middle	K/β5	SRS-5	A330	G388	F382	F382	F396	F396	F375	**L396**	F396	F396	**L396**	F396	F396	**L396**	F396
Middle	β8		M354	L412	T405	**S405**	A419	A419	A398	A419	A419	A419	A419	A419	A419	A419	A419
Middle	β10/β11	SRS-6	L437	L494	A489	**I489**	A503	A503	A482	A503	A503	A503	A503	A503	A503	A503	A503
Middle	β11	SRS-6	T438	V495	I490	I490	T504	T504	T483	T504	T504	T504	T504	T504	T504	T504	T504
Lower	B'	SRS-1	L75	Y120	Y116	Y116	Y118	Y118	Y118	Y118	Y118	Y118	Y118	Y118	Y118	Y118	Y118
Lower	B'/C	SRS-1	F87	L132	L128	L128	L130	L130	L130	L130	L130	L130	L130	L130	L130	L130	L130
Lower	I	SRS-4	T260	T317	T309	T309	T323	T323	T302	T323	T323	T323	T323	T323	T323	T323	T323
Lower	I	SRS-4	F261	F318	F310	F310	F324	F324	F303	F324	F324	F324	F324	F324	F324	F324	F324
Lower	I	SRS-4	A264	E321	E313	**A313**	E327	E327	E306	E327	E327	E327	E327	E327	E327	E327	E327
Lower	I	SRS-4	G265	G322	G314	G314	G328	G328	G307	G328	G328	G328	G328	G328	G328	G328	G328
Lower	I	SRS-4	T268	T325	T317	T317	T331	T331	T310	T331	T331	T331	T331	T331	T331	T331	T331
Lower	K/β5	SRS-5	A328	V386	V380	V380	V394	V394	V373	V394	V394	V394	V394	V394	V394	V394	V394

Upper, Middle and Lower indicate a residue in the upper (near surface), middle or lower (near the active site) region in the binding channel.
Bold residues indicate variations at corresponding positions among *Cyphoma* proteins.
White horizontal bars indicate positions containing the same residues in CYP102, CYP4A11 and *Cyphoma* CYP4s.

Table 10. Leukotriene B_4 hydroxylase activity of recombinant *C. gibbosum* CYP4 proteins.

Clone	Protein	Induction (hrs)[a]	Activity at 23°C (pmol/mg/min)	Activity at 30°C (pmol/mg/min)	Catalytic activity (pmol/min/nmol P450)[b]
Control	--	8	nd	nd	
198_58	CYP4-2a (CYP4BK1)	8	nd	nd	
198_27	CYP4-2b (CYP4BK2)	8	nd	nd	
197_52	CYP4-3a1 (CYP4BL1)	8	nd	0.0173	0.146
197_48	CYP4-3b1 (CYP4BL4)	8	nd	nd	
197_53	CYP4-3b3 (CYP4BL3)	8	0.1180	0.1231	4.40[c]
Control	--	15	nd	nd	
198_58	CYP4-2a (CYP4BK1)	15	nd	nd	
198_27	CYP4-2b (CYP4BK2)	15	nd	nd	
197_52	CYP4-3a1 (CYP4BL1)	15	0.1357	0.2737	n/a
197_48	CYP4-3b1 (CYP4BL4)	15	nd	nd	
197_53	CYP4-3b3 (CYP4BL3)	15	0.1827	0.2996	n/a

All values reported are the average of two technical replicates; nd (not detected)

[a] Indicates time (in hours) yeast were induced on galactose media

[b] Values not applicable (n/a) due to the lack of detection of a peak at 450 nm in P450 content assays

[c] Average of values at 23°C and 30°C

Table 11. Leukotriene B_4 hydroxylase activity in *Cyphoma* microsomes

Cyphoma individual	Diet	Activity (pmol/mg/min)[a]
1	Control	nd
2	Control	nd
3	Control	nd
4	Control	0.40
5	Control	nd
6	Control	nd
7	Control	nd
8	Control	nd
9	Control	nd
10	Control	nd
11	*P. homomalla*	nd
12	*P. homomalla*	1.57
13	*P. homomalla*	0.20
14	*P. homomalla*	0.11

[a]Reporting the average activity of two technical replicates; nd (not detected)
Detection limit of the assay is 0.008 pmol/mg/min

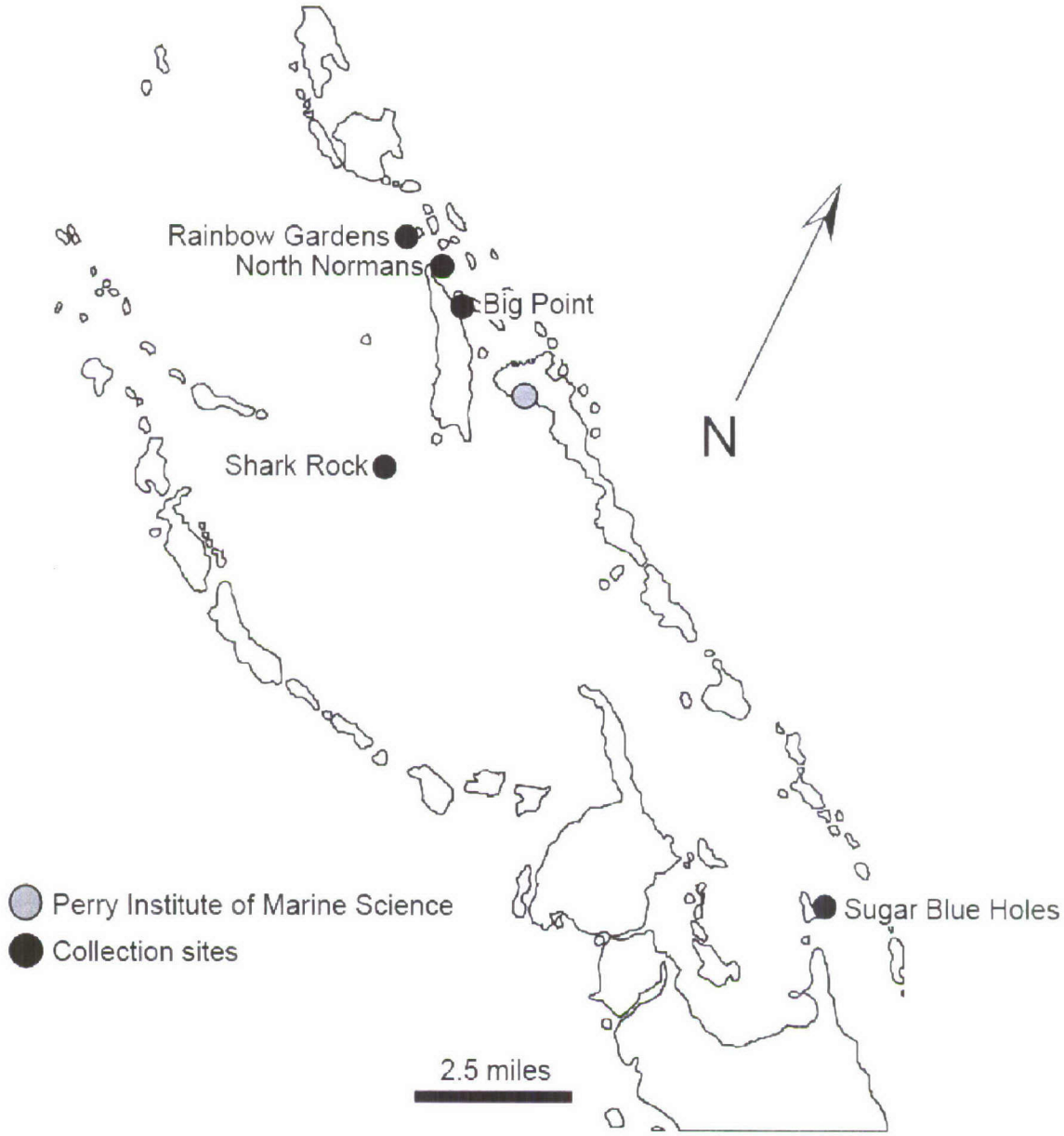


Figure 1. *Cyphoma gibbosum* and gorgonian sampling location in the Exuma Keys, Bahamas. Black circles indicate the locations of reefs where *C. gibbosum* and gorgonian colonies were collected for feeding assays in January of 2004 and 2006.

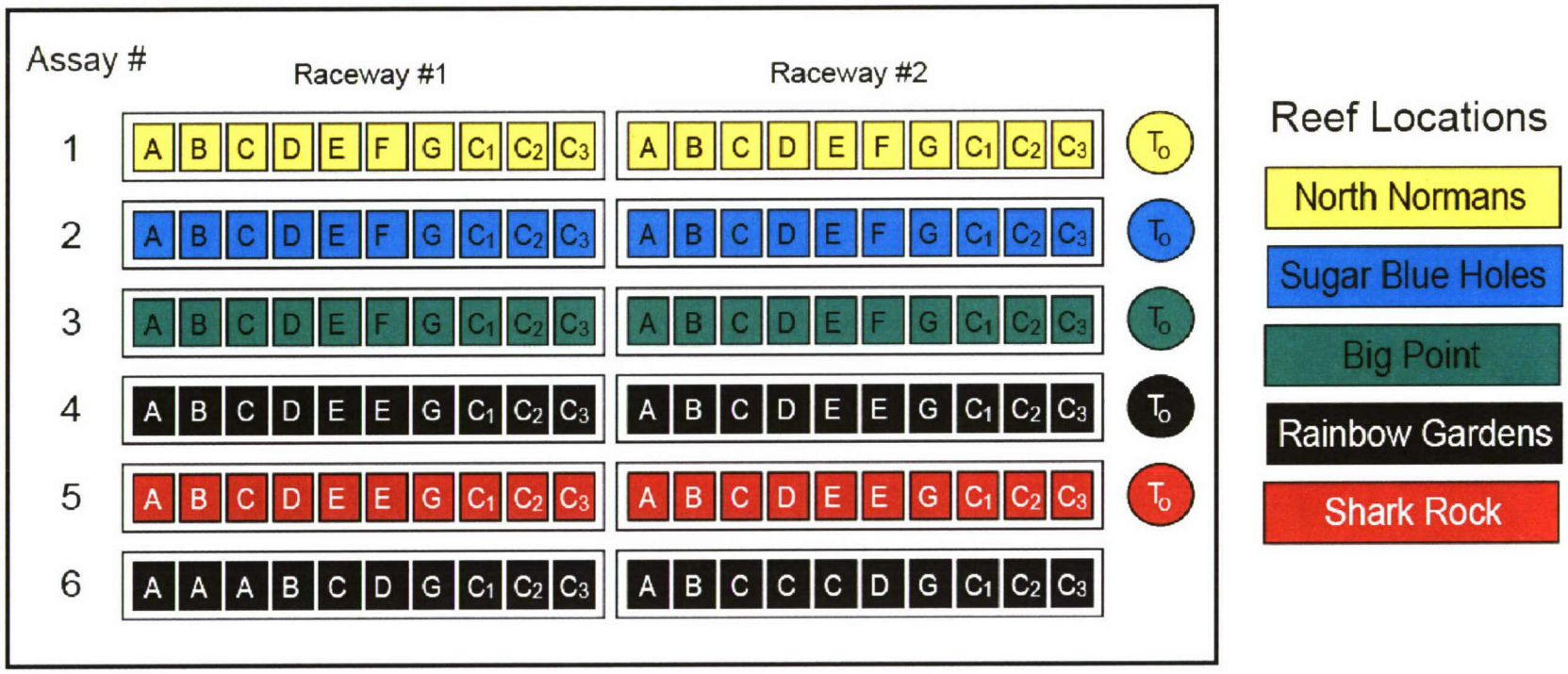


Figure 2. Feeding assay experimental design for snails used for RNA isolation. Snails were randomly placed into one of nine groups, time zero group (T_o), a control group (C_1,C_2, C_3), or one of seven gorgonian diet groups (A, *Briareum asbestinum*; B, *Eunicea mammosa*; C, *Gorgonia ventalina*; D, *Pseudopterogoria acerosa*; E, *Pseudopterogoria americana*; F, *Pseudopterogoria elisabethae*; G, *Plexaura homomalla*). Digestive glands collected after completion of this feeding assay were used for RNA isolation and mRNA expression analysis.

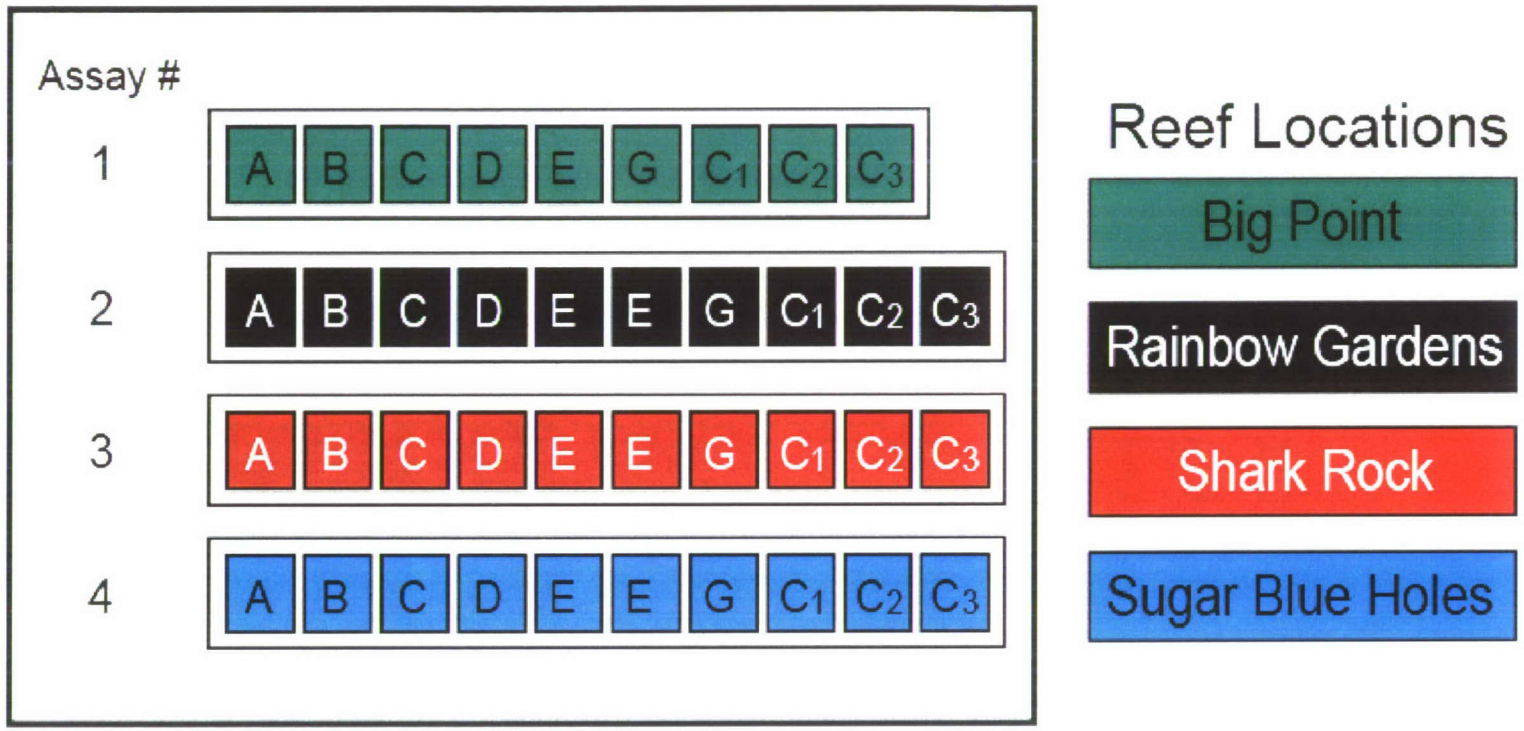


Figure 3. Feeding assay experimental design for snails used for protein isolation. Snails were randomly placed into one of seven groups, a control group (C_1,C_2, C_3), or one of six gorgonian diet groups (A, *Briareum asbestinum*; B, *Eunicea mammosa*; C, *Gorgonia ventalina*; D, *Pseudopterogoria acerosa*; E, *Pseudopterogoria americana*; G, *Plexaura homomalla*). Digestive glands collected after completion of this feeding assay were used for protein analysis and enzymatic characterization.

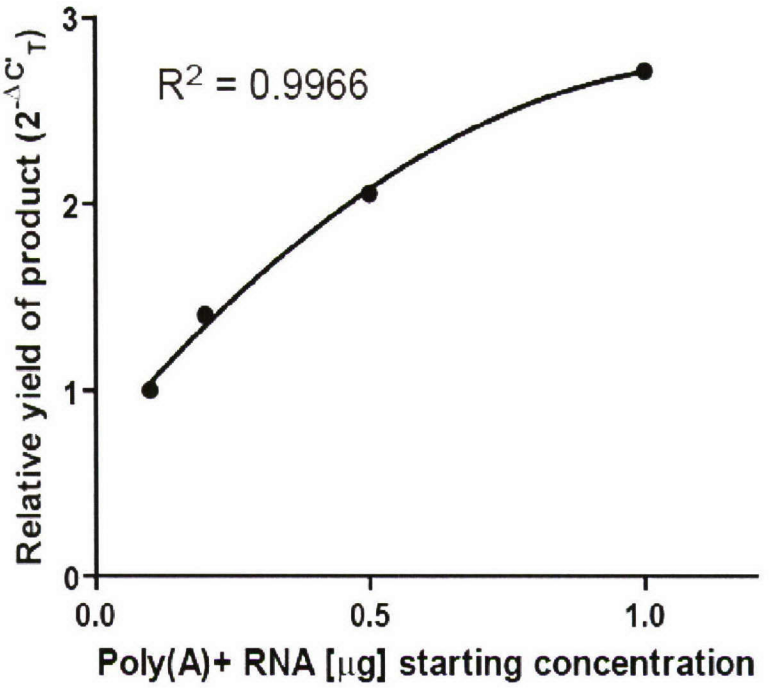


Figure 4. Quantifying relative yield of a target gene by real-time quantitative PCR (qPCR) versus input poly(A)+ RNA concentration. Four concentrations of poly(A)+RNA isolated from a single individual were reverse transcribed to cDNA as described in the methods. Quantitative PCR was performed with CYP4-$3_{(subB)}$ primers as described in the methods. The average ΔC_T was calculated from duplicate qPCR wells for each poly(A)+ RNA concentration and subtracted from the ΔC_T obtained with 1.0 μg poly(A)+RNA yielding $\Delta\Delta C_T$. The $\Delta\Delta C_T$ were then backtransformed ($2^{-\Delta\Delta CT}$) to calculate relative abundance of each sample. A five-fold increase in poly(A)+RNA (0.2 μg → 1.0 μg) quantity affords less than a 1.25x increase in product yield. It was decided to use 0.2 μg of poly(A)+RNA in subsequent qPCR experiments to maximize the yield of cDNA using the least amount of starting poly(A)+RNA.

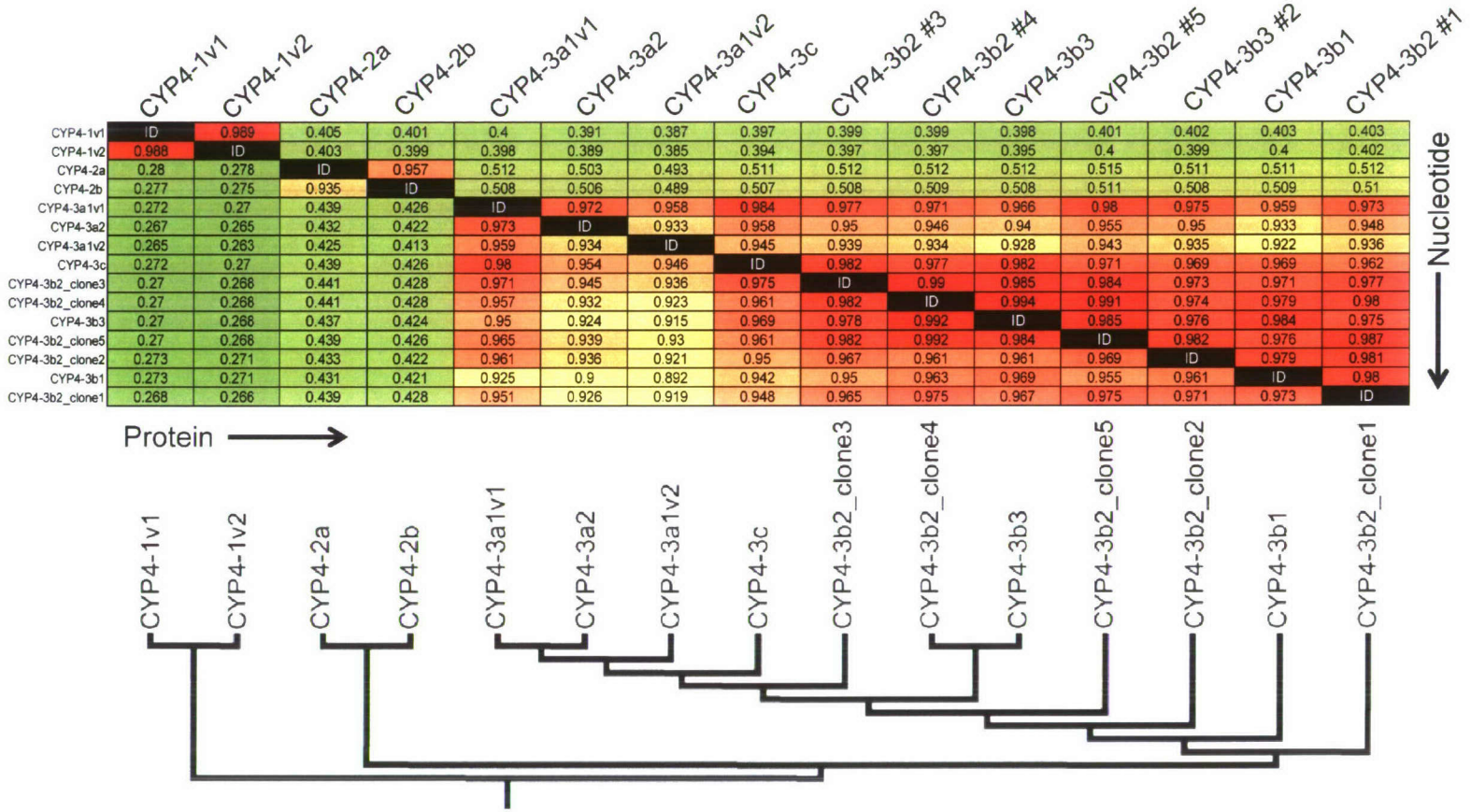


Figure 5. Percent identity matrix for comparison of *Cyphoma* CYP4 nucleotide and amino acid sequences. Values are shaded according to their percent identity with red indicating a high degree of identity between the two sequences. Deduced amino acid sequences were aligned using Clustal X and nucleotide (top) and protein (bottom) percent identities were obtained from BioEdit 7.0.5.2. All positions were compared directly for each pair of sequences one at a time; positions where both sequences have a gap do not contribute to the overall identity; positions where there is a residue in one sequence and a gap in the other do count as a mismatch; the reported number represents the ratio of identities to the length of the longer of the two sequences after positions were both sequences contain a gap are removed. The maximum likelihood tree was constructed from nucleotide consensus sequences as described in the Methods.

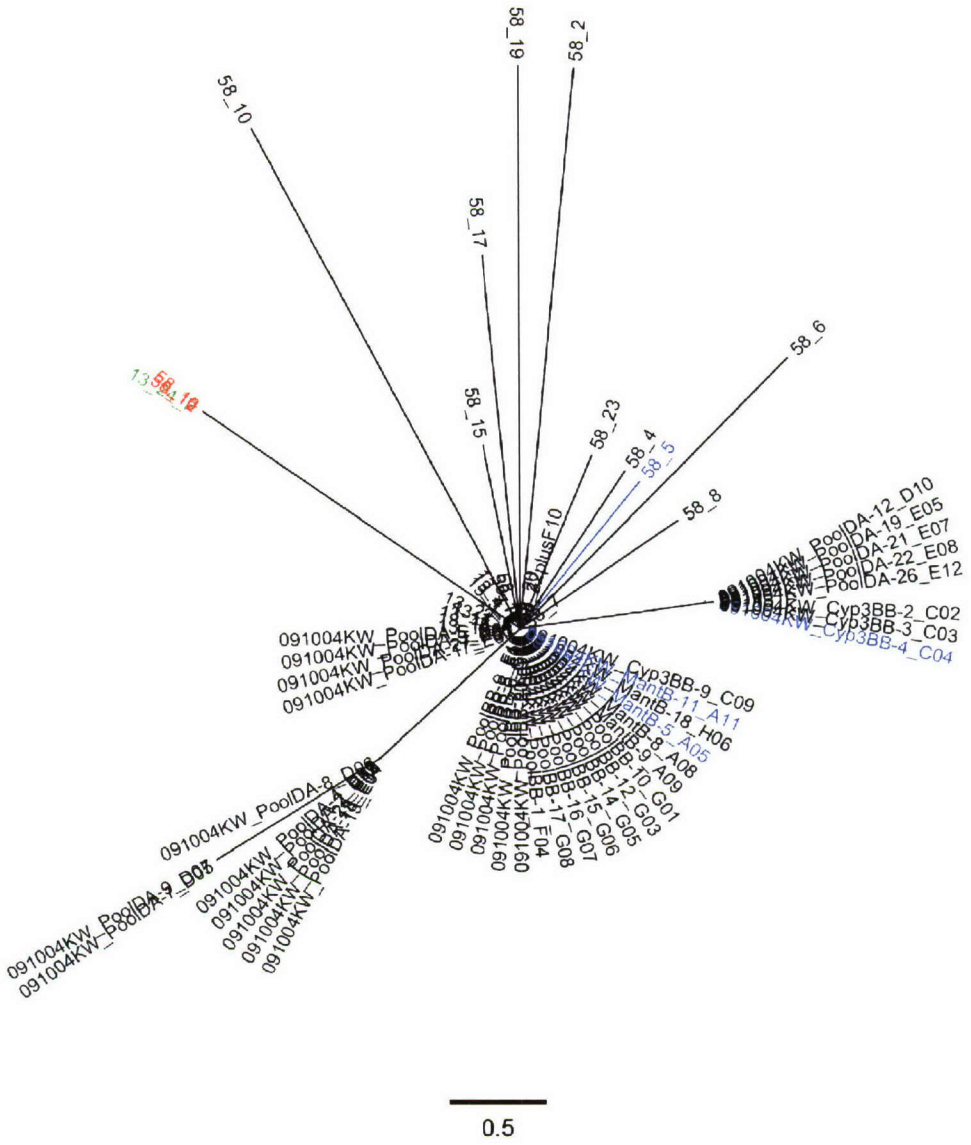


Figure 6. Cluster analysis of CYP4-1 clones visualized by an unrooted maximum parsimony tree. Nucleotide sequences were aligned using ClustalX (Thompson et al., 1997) and the alignment was used to construct a maximum parsimony tree using PAUP*4.0b10 (Swofford 1998). Trees were visualized and manipulated using FigTree v1.1 (Rambaut 2008). Each operational taxonomic unit (OTU) represents the consensus sequence of an individual clone sequenced in both directions. Black OTUs correspond to CYP4-1v1. Red OTUs correspond to CYP4-1v2 and indicate clones with an insertion of 15bp at nucleotide position 1201. Blue OTUs indicate clones with a 68 bp deletion at nucleotide position 1066 causing a frame shift resulting in a premature stop codon likely resulting in a non-functional protein; no designation was given. The green OTU denotes a single clone found to have both the 15 bp insertion and 68 bp deletion. Scale bar indicates the number of nucleotide base changes.



Figure 7. Nucleotide alignment of full-length *Cyphoma gibbosum* CYP4-1v1 and CYP4-1v2. Consensus nucleotide sequence for CYP4-1v1 and allelic variant (CYP4-1v2) are shown and single nucleotide polymorphisms are indicated according to IUPAC naming conventions. Grey boxes and dots within the alignment indicate areas of identical sequence between CYP4-1v1 and CYP4-1v2. Dashes indicate gaps in the alignment. Real-time quantitative PCR primers locations are indicated by arrows. GenBank Accession numbers: CYP4-1v1 (CYP4V10v1, *EU546250*), CYP4-1v2 (CYP4V10v2, *EU546251*).

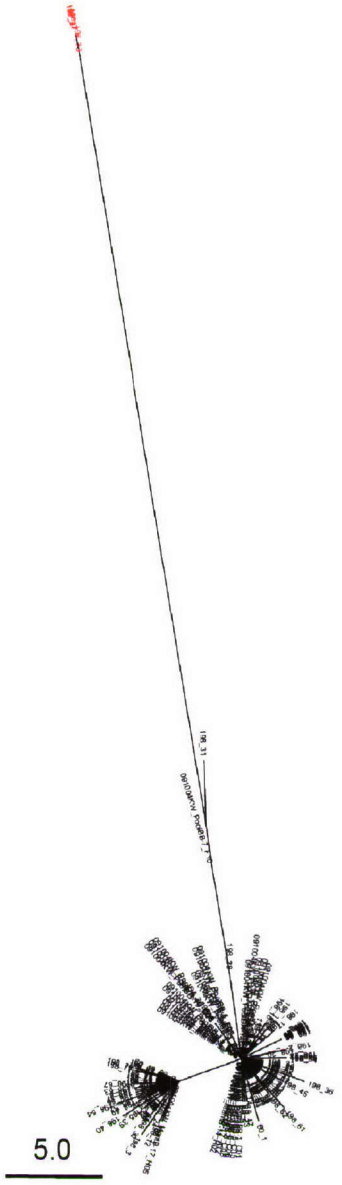


Figure 8. Cluster analysis of CYP4-2 clones visualized by an unrooted maximum parsimony tree. Nucleotide sequences were aligned using ClustalX (Thompson et al., 1997) and the alignment was used to construct a maximum parsimony tree using PAUP*4.0b10 (Swofford 1998). Trees were visualized and manipulated using FigTree v1.1 (Rambaut 2008). Each operational taxonomic unit (OTU) represents the consensus sequence of an individual clone sequenced in both directions. Black OTUs correspond to CYP4-2b cDNAs and red OTUs (4 clones) correspond to CYP4-2a cDNAs. Scale bar indicates the number of nucleotide base changes.

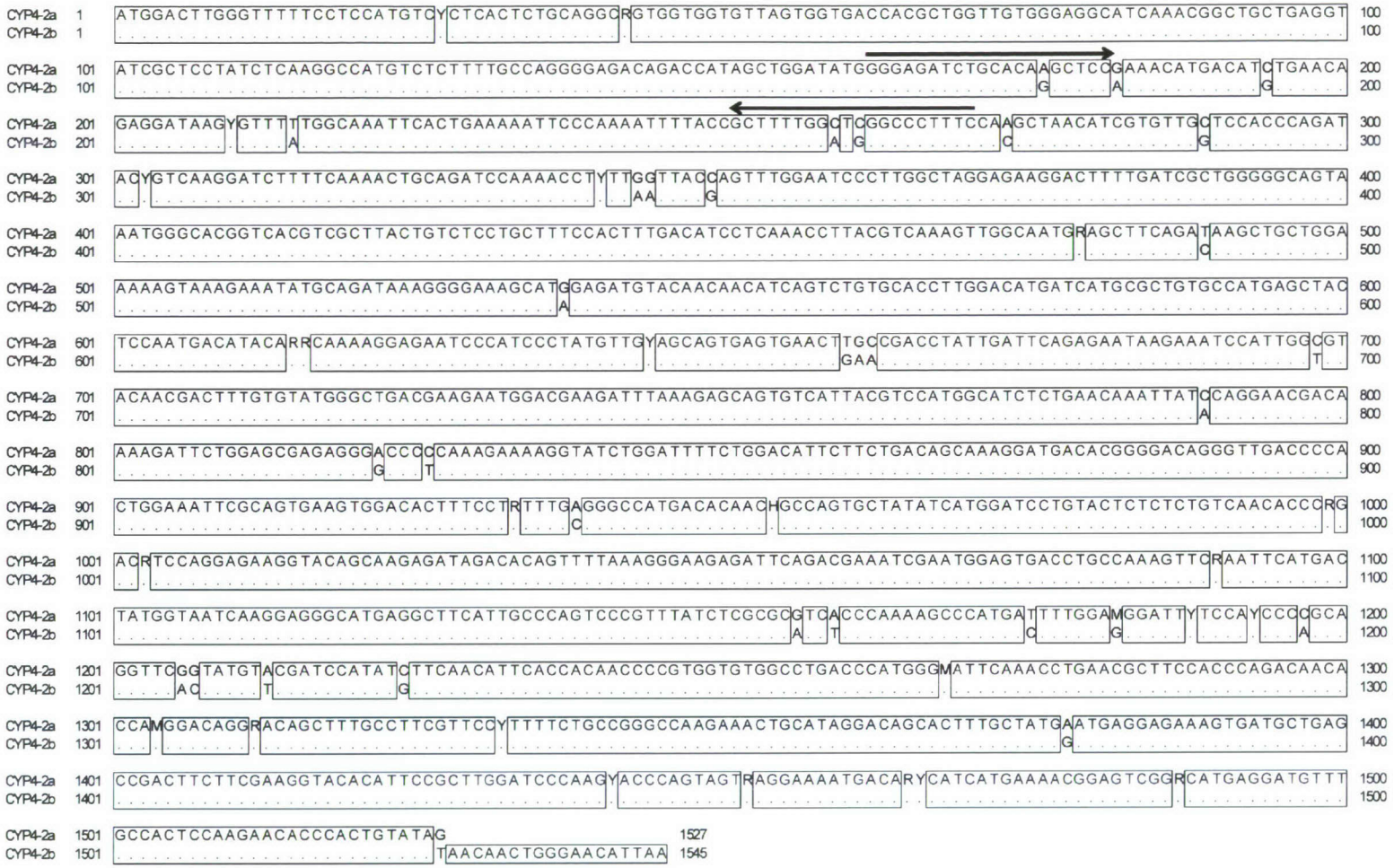


Figure 9. Nucleotide alignment of full-length *Cyphoma gibbosum* CYP4-2a and CYP4-2b. The consensus nucleotide sequences for both CYP4-2 cDNAs (CYP4-2a and CYP4-2b) are shown and nucleotide polymorphisms are indicated according to IUPAC naming conventions. Grey boxes and dots in the alignment indicate areas of identical sequence between both cDNAs. Real-time quantitative PCR primer locations are indicated by arrows. GenBank Accession numbers: CYP4-2a (CYP4BK1, *EU546252*); CYP4-2b (CYP4BK2, *EU546253*).

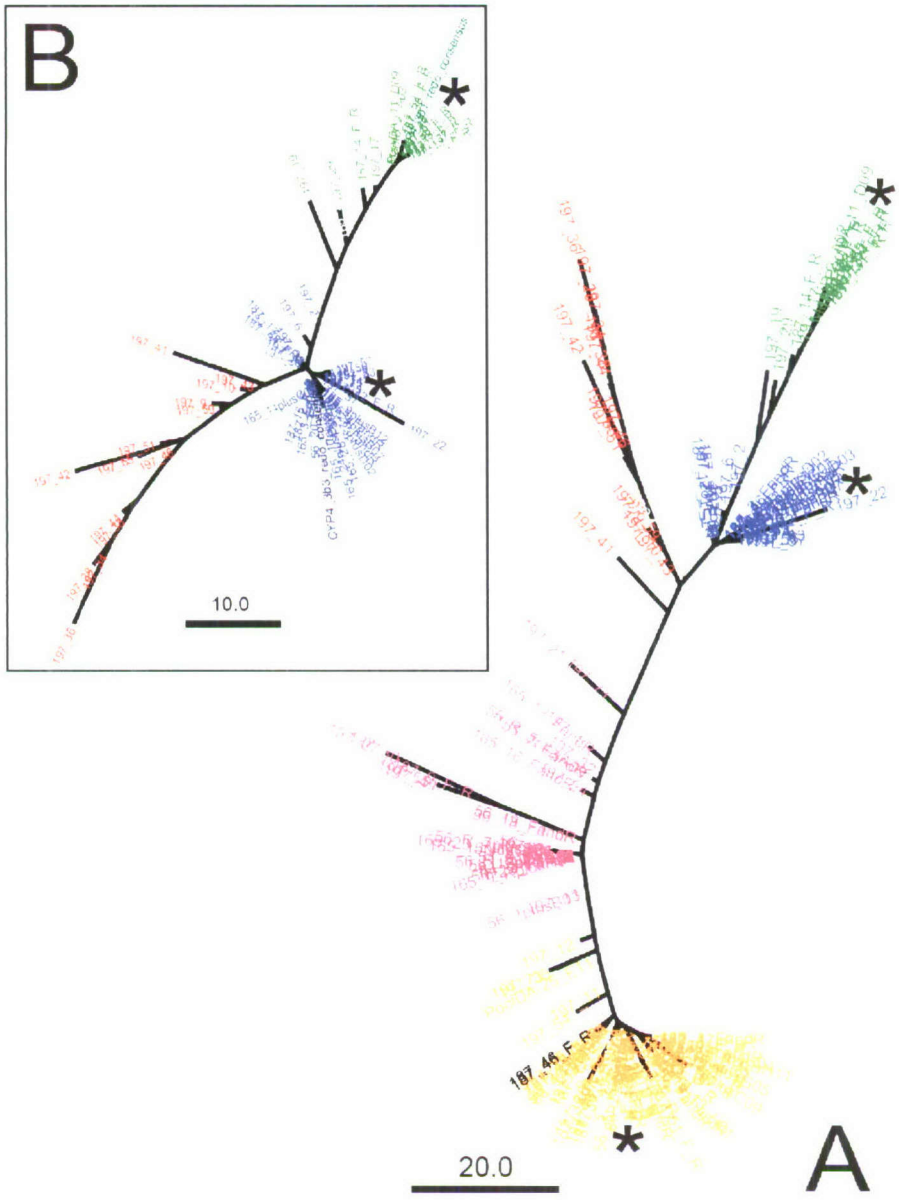
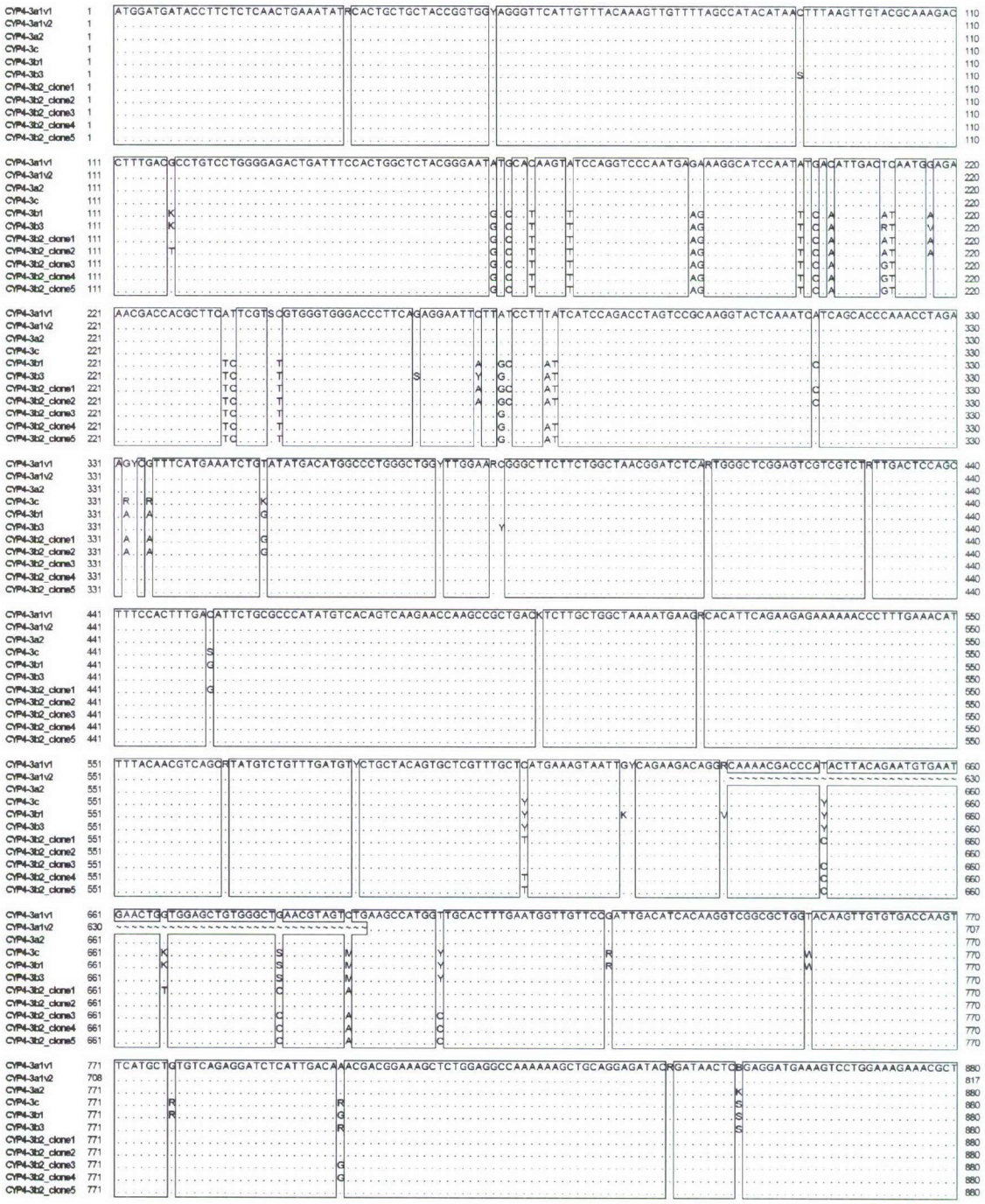
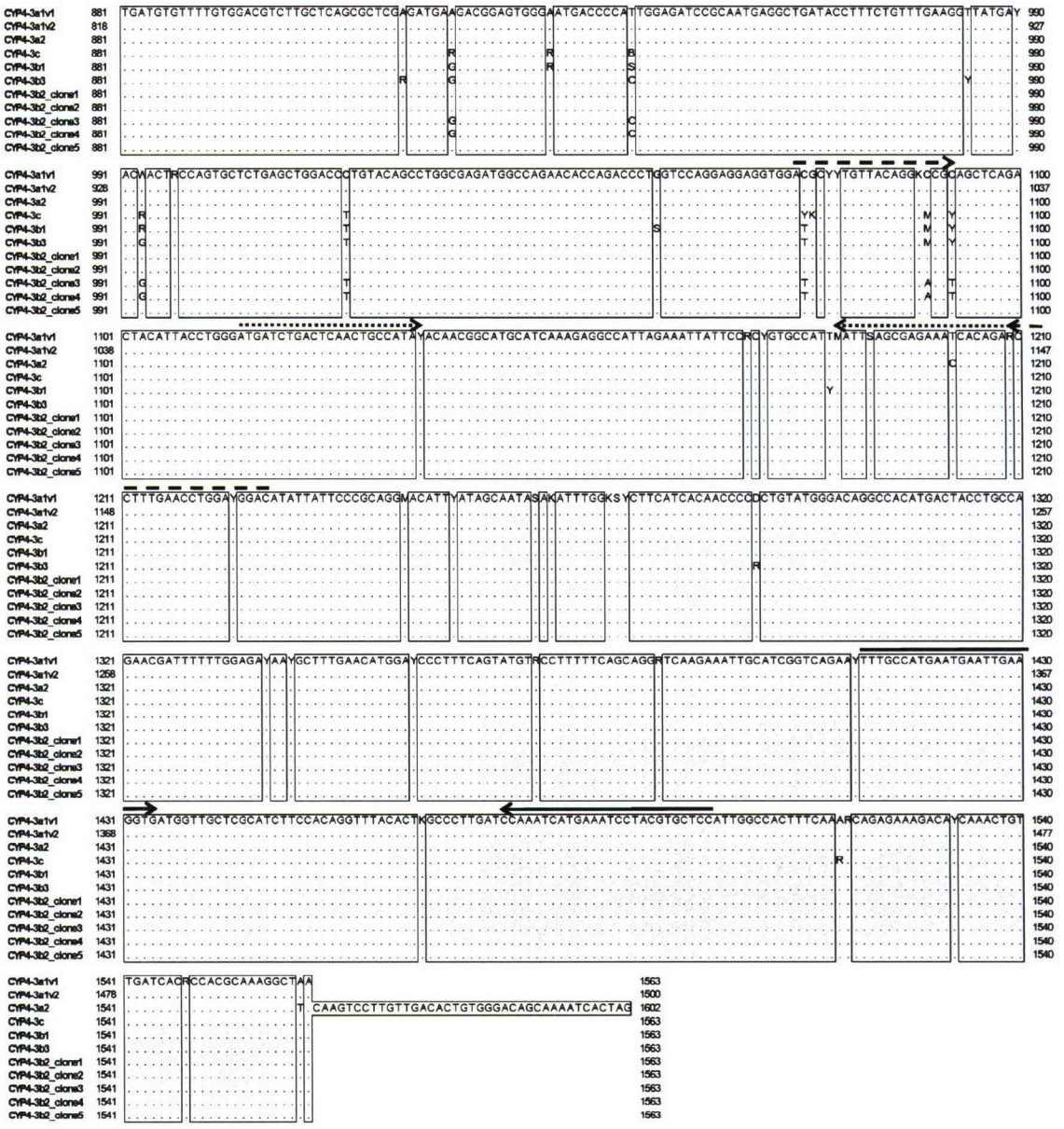


Figure 10. Cluster analysis of CYP4-3 clones visualized by an unrooted maximum parsimony tree. Nucleotide sequences were aligned using ClustalX (Thompson et al., 1997) and the alignment was used to construct a maximum parsimony tree using PAUP*4.0b10 (Swofford 1998). Trees were visualized and manipulated using FigTree v1.1 (Rambaut 2008). Each operational taxonomic unit (OTU) represents the consensus sequence of an individual clone sequenced in both directions. The color of the OTU indicates further groupings denoting possible distinct loci. Part (**A**) depicts all clones within the CYP4-3 grouping; CYP4-3a1=orange, CYP4-3a2=black, CYP4-3b1=green, CYP4-3b2=red, CYP4-3b3=blue, CYP4-3c=purple. Part (**B**) is an expanded view of the CYP4-3b node. Clones used in yeast heterologous expression experiments are indicated by an asterisk. Scale bar indicates the number of nucleotide base changes.

Figure 11. Nucleotide alignment of eleven full-length *Cyphoma gibbosum* CYP4-3 cDNA sequences. The consensus nucleotide sequences for all eleven cDNAs, including possible allelic variants, are shown. Nucleotide polymorphisms are indicated according to IUPAC naming conventions. CYP4-3a sequences are represented three allelic variants. CYP4-3b2 sequences are represented by five individual clones because no 'physical' clones could be supported by the consensus sequence generated for the CYP4-3b2 subgroup. Identical bases are indicated with a "•" and are shaded grey. Real-time quantitative PCR primer locations are indicated by arrows; solid arrows correspond to all CYP4-3 sequences; dashed arrows correspond to CYP4-$3_{(sub\ A)}$ sequences; dotted arrows correspond to CYP4-$3_{(sub\ B)}$ sequences. GenBank Accession numbers: CYP4-3a1v1 (CYP4BL1v1, *EU546254*); CYP4-3a1v2 (CYP4BL1v2, *EU546255*); CYP4-3a2 (CYP4BL1v3, *EU546256*); CYP4-3b1 (CYP4BL4, *EU546257*); CYP4-3b2_clone1 (CYP4BL5, *EU546258*); CYP4-3b2_clone2 (CYP4BL6, *EU546259*); CYP4-3b2_clone3 (CYP4BL7, *EU546260*); CYP4-3b2_clone4 (CYP4BL8, *EU546261*); CYP4-3b2_clone5 (CYP4BL9, *EU546262*); CYP4-3b3 (CYP4BL3, *EU546263*).


CYP4-3a1v1
CYP4-3a1v2
CYP4-3a2
CYP4-3c
CYP4-3b1
CYP4-3b3
CYP4-3b2_clone1
CYP4-3b2_clone2
CYP4-3b2_clone3
CYP4-3b2_clone4
CYP4-3b2_clone5


CYP4-3a1v1
CYP4-3a1v2
CYP4-3a2
CYP4-3c
CYP4-3b1
CYP4-3b3
CYP4-3b2_clone1
CYP4-3b2_clone2
CYP4-3b2_clone3
CYP4-3b2_clone4
CYP4-3b2_clone5

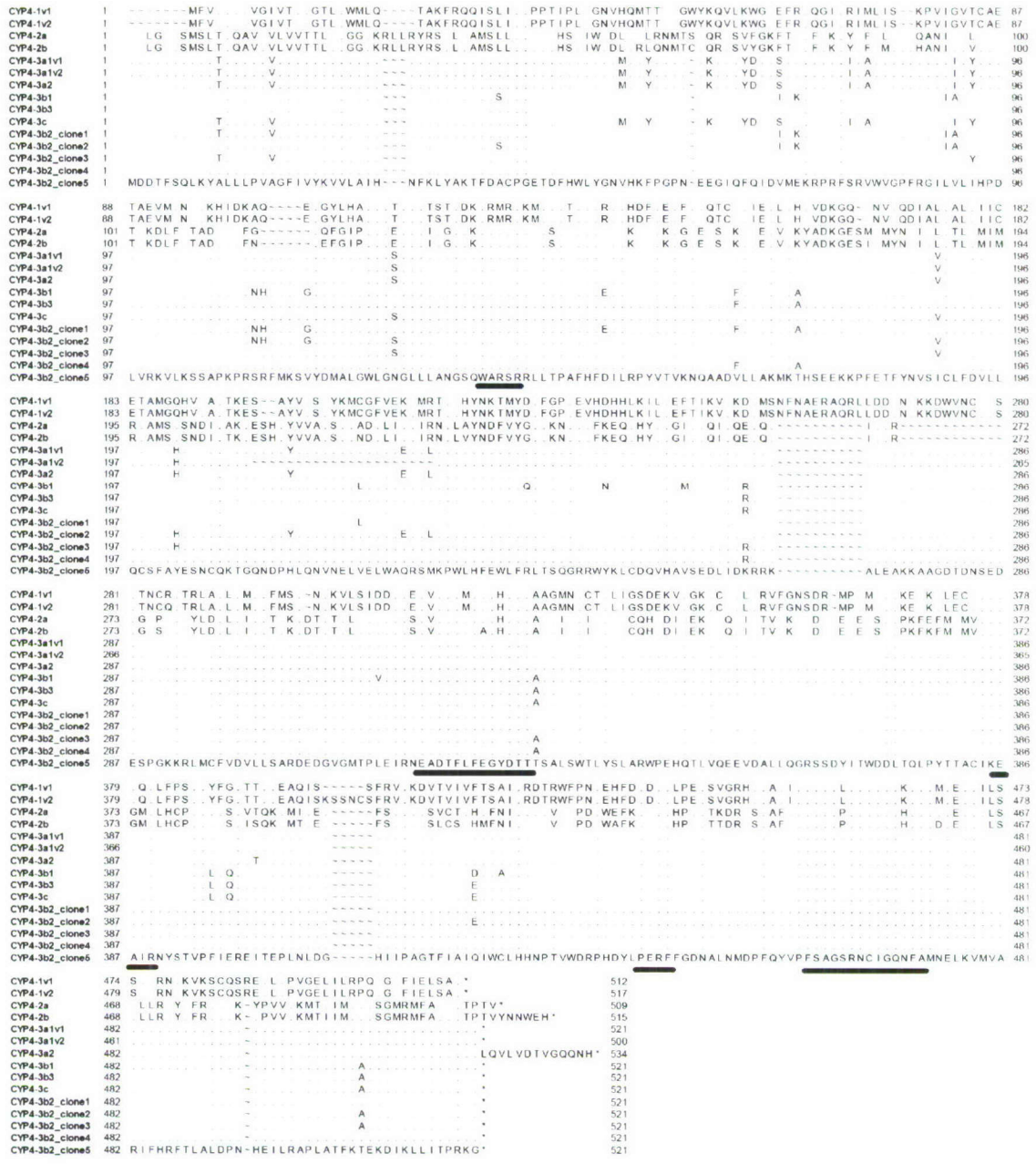
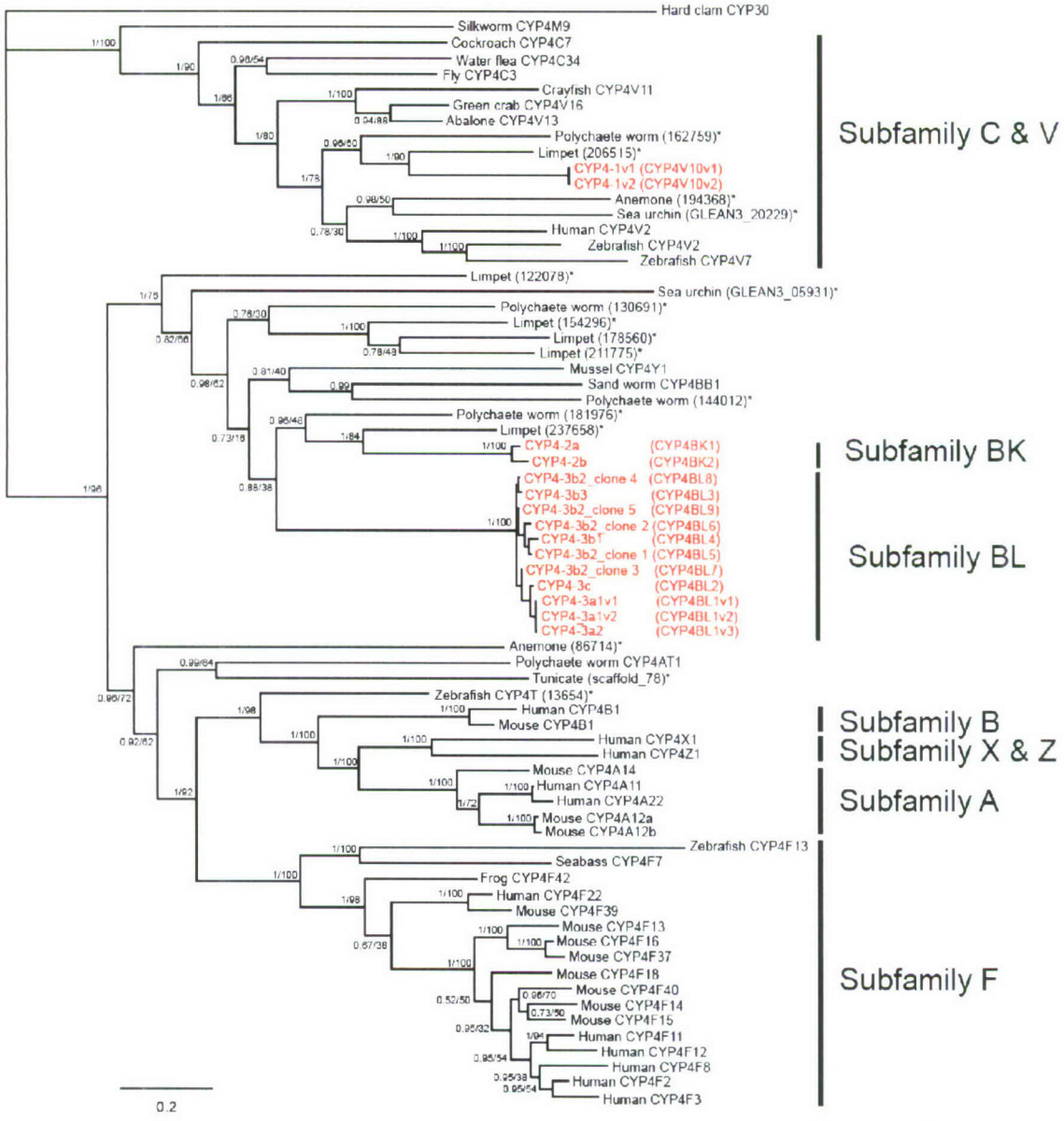


Figure 12. Deduced amino acid alignment of *C. gibbosum* CYP4-1, CYP-4-2, and CYP4-3 consensus sequences. Amino acid sequences were generated from consensus nucleotide sequences and aligned using ClustalX. Grey boxes indicate areas of identical sequence among all cDNAs. Amino acid residues identical to CYP4-3b2_clone 5 are indicated by a "•" and gaps by a "~". The stop codon is denoted by an asterisk. The conserved WXXXR, I-helix, EXXR, PXRF, and heme-binding domains, in order, are indicated by black lines under the sequence alignment.

Figure 13. Phylogenetic relationships of *Cyphoma gibbosum* CYP4 proteins with other invertebrate and vertebrate CYP4s. Bayesian and Maximum Likelihood (ML) trees were constructed as described in the Methods. Both trees resulted in the same topology, and the Bayesian tree is depicted here. Sequences from *C. gibbosum* are highlighted in red. Values at branch points represent posterior probabilities derived from $3x10^6$ generations and ML bootstrap values calculated with 1000 replications. Predicted sequences are labeled with an asterisk. Sequences include: Abalone CYP4V13 formally CYP4C17 (*H. rufescens*, AAC32833), Anemone (*N. vectensis*, JGI # 194368), Anemone (*N. vectensis*, JGI # 86714), Cockroach CYP4C7 (*D. punctata*, AAC69184), Crayfish CYP4V11 formally CYP4C15 (*O. limosus*, AAF09264), Fly CYP4C3 (*D. melanogaster*, FlyBase FBpp0085074), Frog CYP4F42 (*X. laevis*, BAD02914.1), Green crab CYP4V16 formally CYP4C39 (*C. maenas*, JC8026), Hard clam CYP30 (*M. mercenaria*, AAB66556), Human CYP4A11 (NP_000769.2), Human CYP4A22 (NP_001010969.2), Human CYP4B1 (NP_000770.2), Human CYP4F11 (NP_067010.2), Human CYP4F12 (NP_076433.2), Human CYP4F2 (XP_001172541.1), Human CYP4F22 (NP_775754.2), Human CYP4F3 (NP_000887.2), Human CYP4F8 (NP_009184.1), Human CYP4V2 (NP_997235.2), Human CYP4X1 (NP_828847.1), Human CYP4Z1 (NP_835235.1), Limpet (*L. gigantea*, JGI# 122078), Limpet (*L. gigantea*, JGI# 154296), Limpet (*L. gigantea*, JGI# 178560), Limpet (*L. gigantea*, JGI# 206515), Limpet (*L. gigantea*, JGI# 211775), Limpet (*L. gigantea*, JGI# 237658), Mouse CYP4A12a (NP_803125.2), Mouse CYP4A12b (NP_758510.2), Mouse CYP4A14 (NP_031848.1), Mouse CYP4B1 (NP_031849.1), Mouse CYP4F13 (NP_570952.1), Mouse CYP4F14 (NP_071879.1), Mouse CYP4F15 (NP_598888.1), Mouse CYP4F16 (NP_077762.1), Mouse CYP4F18 (NP_077764.1), Mouse CYP4F37 (NP_001093657.1), Mouse CYP4F39 (AAI45756), Mouse CYP4F40 (NP_001095058.1), Mussel CYP4Y1 (*M. galloprovincialis*, AAC32835), Polychaete worm (*Capitella sp.* JGI# 130691), Polychaete worm (*Capitella sp.* JGI# 144012), Polychaete worm (*Capitella sp.* JGI# 162759), Polychaete worm (*Capitella sp.* JGI# 181976), Polychaete worm CYP4AT1 (*C. capitata,* AAS87604), Sand worm CYP4BB1 (*N. virens*, AAR88241), Sea urchin (*S. purpuratus*, J. Goldstone predicted, GLEAN3_ 05931), Sea urchin (*S. purpuratus*, J. Goldstone predicted, GLEAN3_20229), Seabass (*D. labrax*, AAD32564), Silkworm CYP4M9 (*B. mandarina*, ABK27872.1), Tunicate (C. intestinalis, JGI# Sc78), Water flea CYP4C34 (*D. pulex*, BQ703383), Zebrafish CYP 4T (*D. rerio*, Ensembl ENSDART00000013654), Zebrafish CYP4F13 (*D. rerio*, Ensembl ENSDART00000063442), Zebrafish CYP4V2 (*D. rerio*, Ensembl ENSDART00000089480), Zebrafish CYP4V7(*D. rerio*, Ensembl ENSDART00000087976). Phylogenetic tree construction by J. Goldstone.


Hard clam CYP30
Silkworm CYP4M9
Cockroach CYP4C7
Water flea CYP4C34
Fly CYP4C3
Crayfish CYP4V11
Green crab CYP4V16
Abalone CYP4V13
Polychaete worm (162759)*
Limpet (206515)*
CYP4-1v1 (CYP4V10v1)
CYP4-1v2 (CYP4V10v2)
Anemone (194368)*
Sea urchin (GLEAN3_20229)*
Human CYP4V2
Zebrafish CYP4V2
Zebrafish CYP4V7
Subfamily C & V
Limpet (122078)*
Sea urchin (GLEAN3_05931)*
Polychaete worm (130691)*
Limpet (154296)*
Limpet (178560)*
Limpet (211775)*
Mussel CYP4Y1
Sand worm CYP4BB1
Polychaete worm (144012)*
Polychaete worm (181976)*
Limpet (237658)*
CYP4-2a (CYP4BK1)
CYP4-2b (CYP4BK2)
Subfamily BK
CYP4-3b2_clone 4 (CYP4BL8)
CYP4-3b3 (CYP4BL3)
CYP4-3b2_clone 5 (CYP4BL9)
CYP4-3b2_clone 2 (CYP4BL6)
CYP4-3b1 (CYP4BL4)
CYP4-3b2_clone 1 (CYP4BL5)
CYP4-3b2_clone 3 (CYP4BL7)
CYP4-3c (CYP4BL2)
CYP4-3a1v1 (CYP4BL1v1)
CYP4-3a1v2 (CYP4BL1v2)
CYP4-3a2 (CYP4BL1v3)
Subfamily BL
Anemone (86714)*
Polychaete worm CYP4AT1
Tunicate (scaffold_78)*
Zebrafish CYP4T (13654)*
Human CYP4B1
Mouse CYP4B1
Subfamily B
Human CYP4X1
Human CYP4Z1
Subfamily X & Z
Mouse CYP4A14
Human CYP4A11
Human CYP4A22
Mouse CYP4A12a
Mouse CYP4A12b
Subfamily A
Zebrafish CYP4F13
Seabass CYP4F7
Frog CYP4F42
Human CYP4F22
Mouse CYP4F39
Mouse CYP4F13
Mouse CYP4F16
Mouse CYP4F37
Mouse CYP4F18
Mouse CYP4F40
Mouse CYP4F14
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Human CYP4F3
Subfamily F
0.2
*denotes predicted sequences

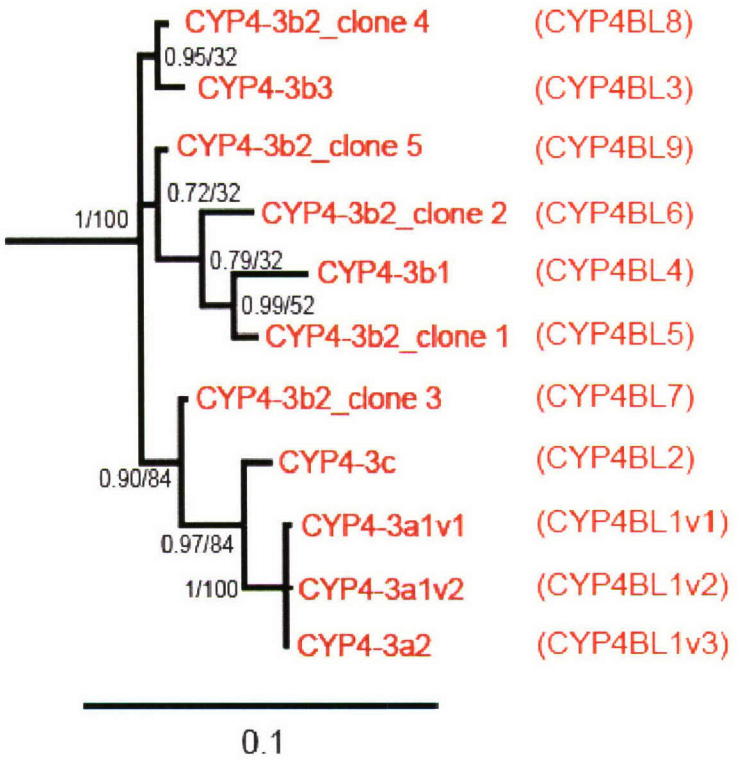


Figure 14. Phylogenetic relationships among *Cyphoma gibbosum* CYP4-3 proteins. Partial tree representing an expanded view of the CYP4-3 node from Figure 13. Values at branch points represent posterior probabilities derived from $3x10^6$ generations and maximum likelihood bootstrap values calculated with 1000 replications. Gene designations assigned by the P450 Nomenclature Committee are indicated to the right of the tree. Phylogenetic tree construction by J. Goldstone.

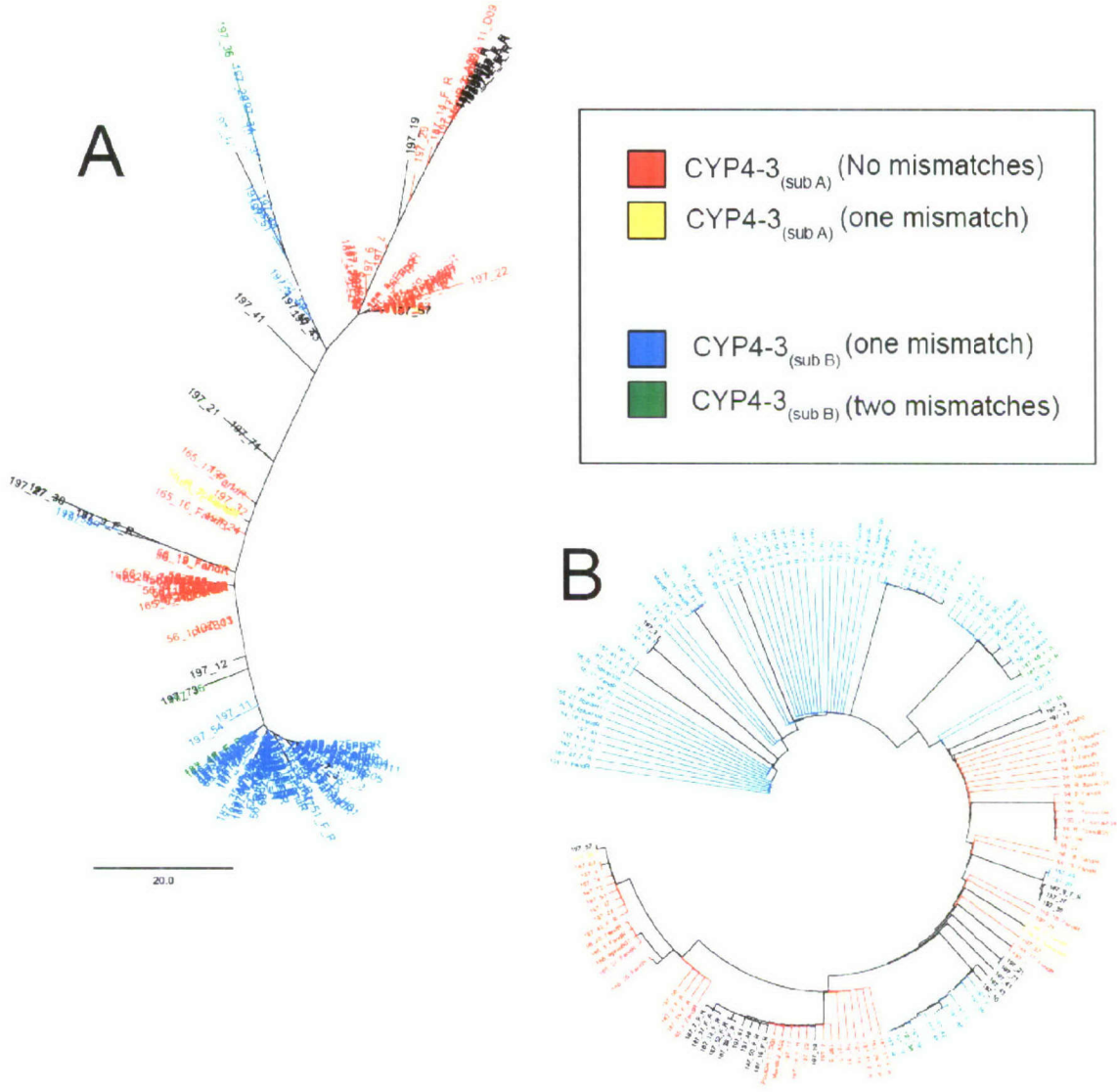


Figure 15. Maximum parsimony trees depicting transcripts recognized by CYP4-3$_{(sub\ A)}$ and CYP4-3$_{(sub\ B)}$ quantitative RT-PCR primers. Each taxon represents the consensus sequence of an individual clone sequenced in both directions. The qPCR primer set used to detect clone-specific abundance is indicated by operational taxonomic unit (OTU) color. OTUs in *red* and *orange* denote those clones that are picked up by CYP4-3$_{(sub\ A)}$ qPCR primers. *Red* OTUs indicate clones that contain no nucleotide mismatches with qPCR primers. *Orange* OTUs (n = 3) indicate clones with a one base pair mismatch in the forward primer, not within three base pairs of the 3' end. OTUs in *blue* and *green* denote those clones that are picked up by CYP4-3$_{(sub\ B)}$ qPCR primers. *Blue* OTUs indicate clones that contain a one base pair mismatch in the forward primer ten base pairs from the 3'end. *Green* OTUs (n = 4) indicate clones with a one base pair mismatch in both the forward and reverse primers not within ten base pairs of the 3' end. Black OTUs denote clones that would not be picked up by either primer set. Partial sequences not covered by the qPCR primer pairs described here were deleted from the above trees. The (**A**) unrooted maximum parsimony tree (see Figure X) was proportionally transformed into a circular tree (**B**) using FigTree v1.1 (Rambaut 2008). The scale bar in (**A**) indicates the number of nucleotide base changes.

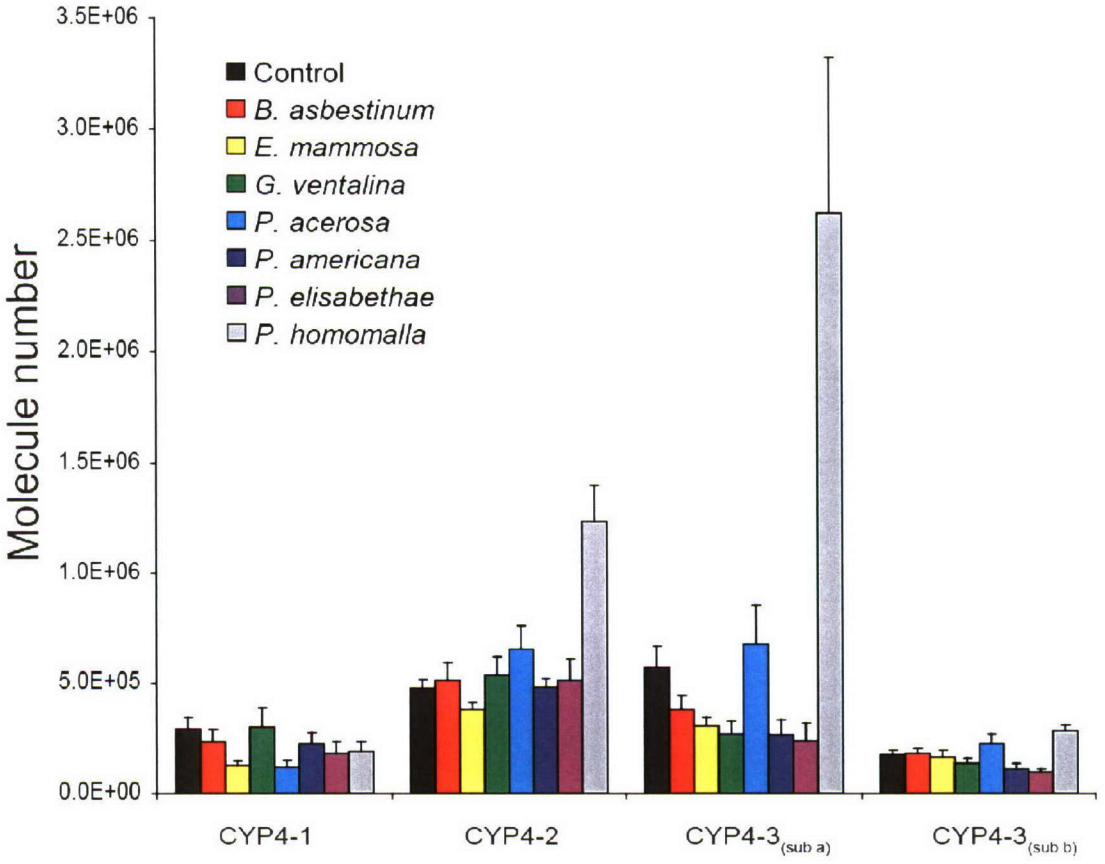


Figure 16. Mean CYP4 gene expression among *C. gibbosum* feeding on gorgonian and control diets for four days. Bars represent the mean CYP4 gene expression (± SE) of snails feeding on *B. asbestinum* (n = 13), *E. mammosa* (n = 12), *G. ventalina* (n = 13), *P. acerosa* (n = 10), *P. americana* (n = 12), *P. elisabethae* (n = 6), *P. homomalla* (n = 11) and the control diet (n = 33). Results from snails collected from all five reefs are shown. Real-time quantitative PCR was performed and the number of molecules per 0.2 μg of polyA+ RNA was calculated from the standard curve and normalized by a β-actin correction factor.

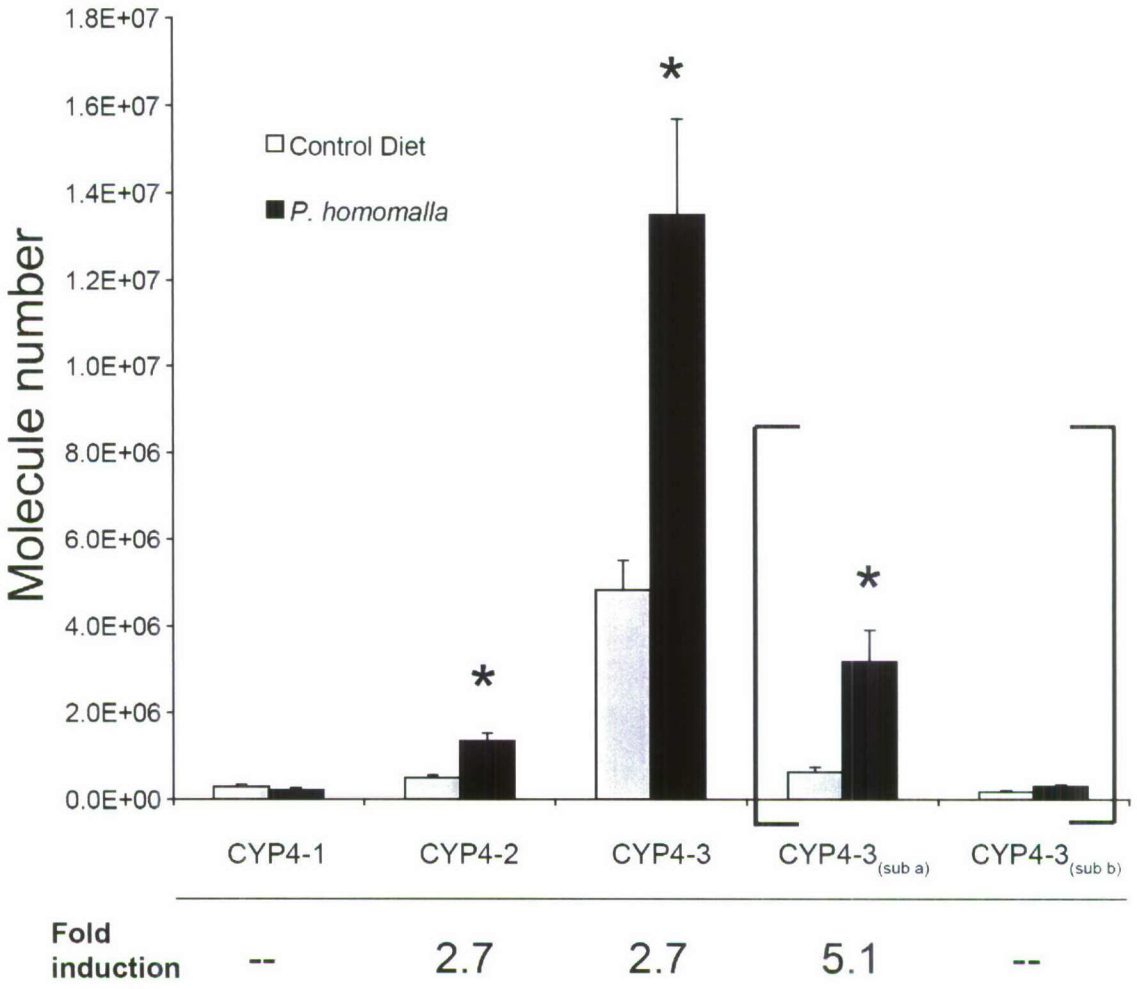


Figure 17. Induction of CYP4 gene expression in *C. gibbosum* feeding on the gorgonian *Plexaura homomalla* compared to diet controls. Bars represent the mean CYP4 gene expression (± SE) of snails feeding on control diet (n = 27 snails; e.g. alginic acid + squid powder) or *P. homomalla* (n = 9 snails) for 4d. Results shown here only include snails from four reefs (Big Point, North Normans, Rainbow Gardens, and Sugar Blue Holes). Real-time quantitative PCR was performed and the number of molecules per 0.2 μg of polyA+ RNA was calculated from the standard curve and normalized by a β-actin correction factor. Bars in the [ ] indicate molecule numbers for a subset of genes within group CYP4-3 (see Figure 15 for more information). Statistically significant induction ($p<0.001$) is indicated by an asterisk.

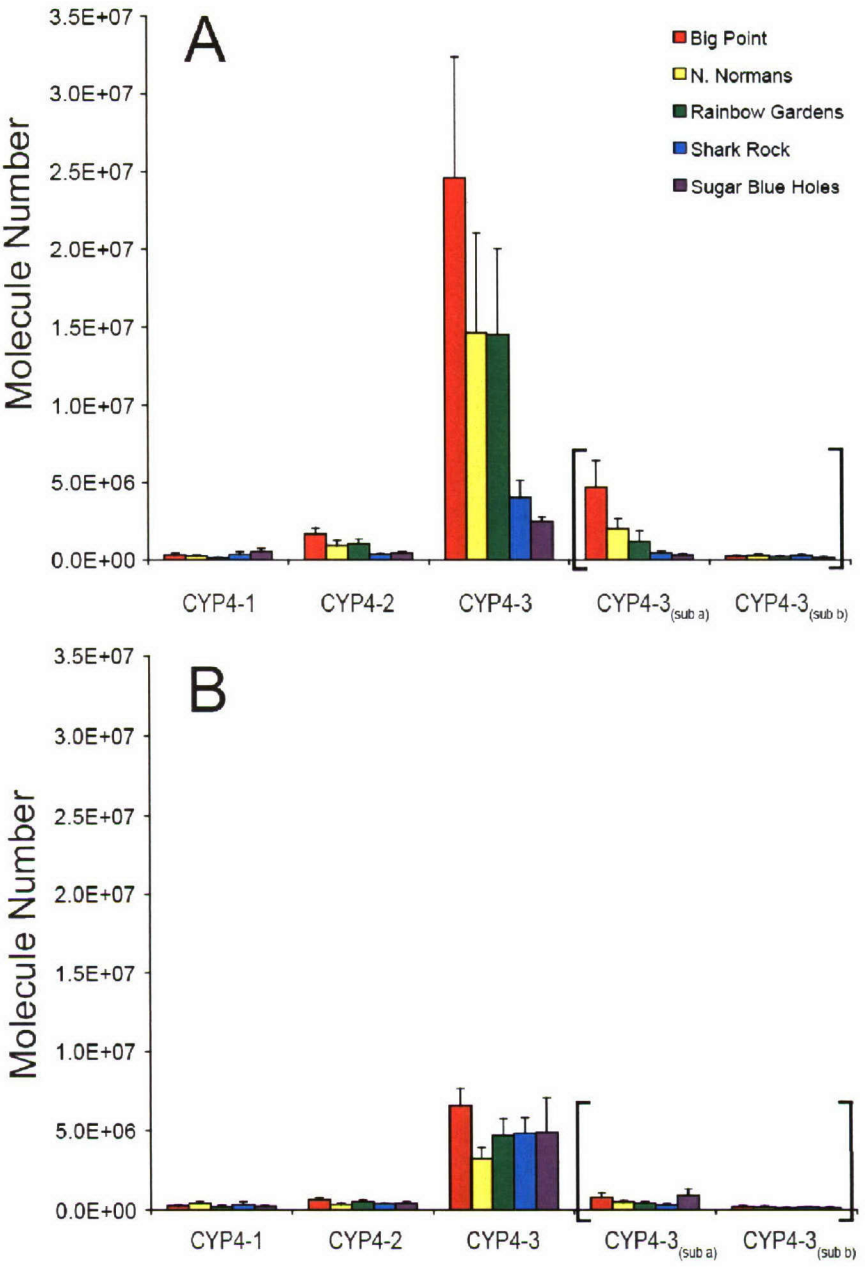


Figure 18. Comparing CYP4 expression levels among time-zero and 4 d control-fed *C. gibbosum* snails collected from five reefs. (**A**) Control snails; Bars represent mean CYP4 expression (± SE) in snails (n = 33) collected from five reefs and fed a control diet (e.g. alginic acid + squid powder) for four days. (**B**) Time-zero snails; Bars represent mean CYP4 expression (± SE) in snails (n = 30) collected from five reefs and immediately dissected to preserve reef-specific gene expression signals. Real-time quantitative PCR was performed and the number of molecules per 0.2 µg of polyA+ RNA was calculated from the standard curve and normalized by a β-actin correction factor. Bars in the [ ] indicate molecule numbers for a subset of genes within group CYP4-3.

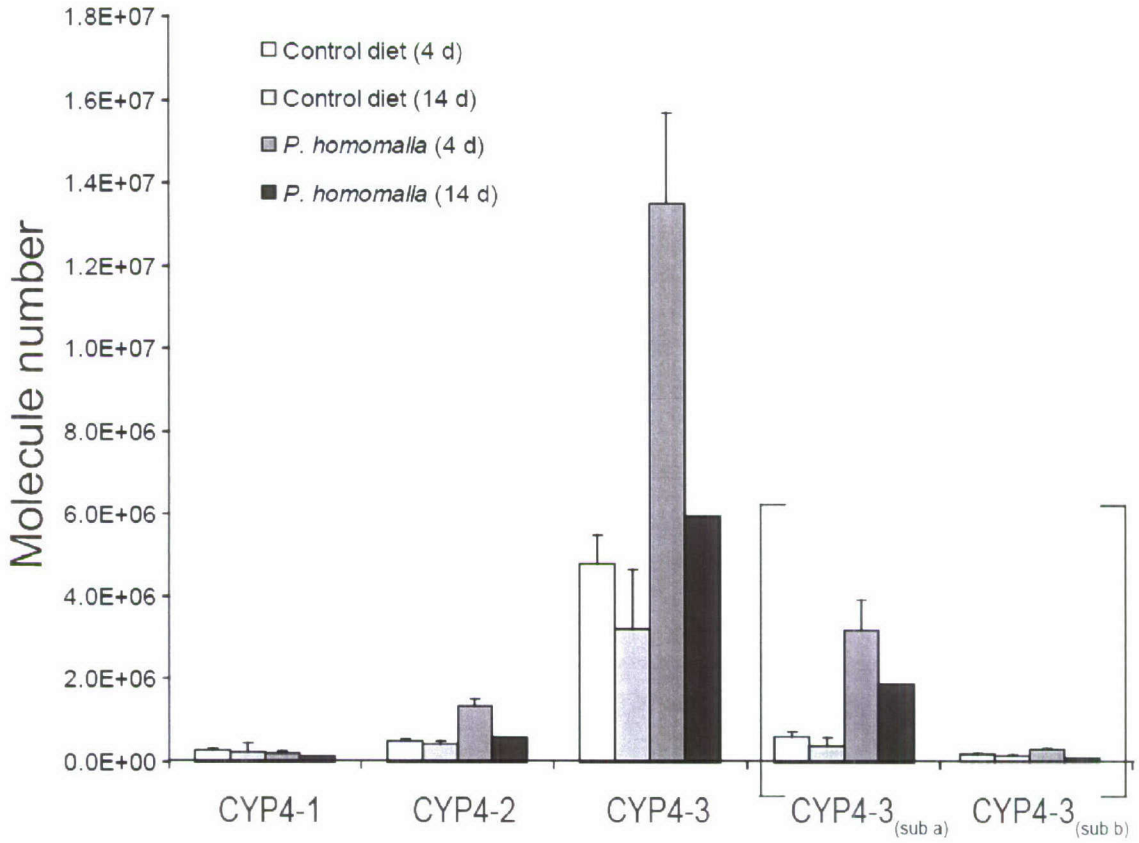
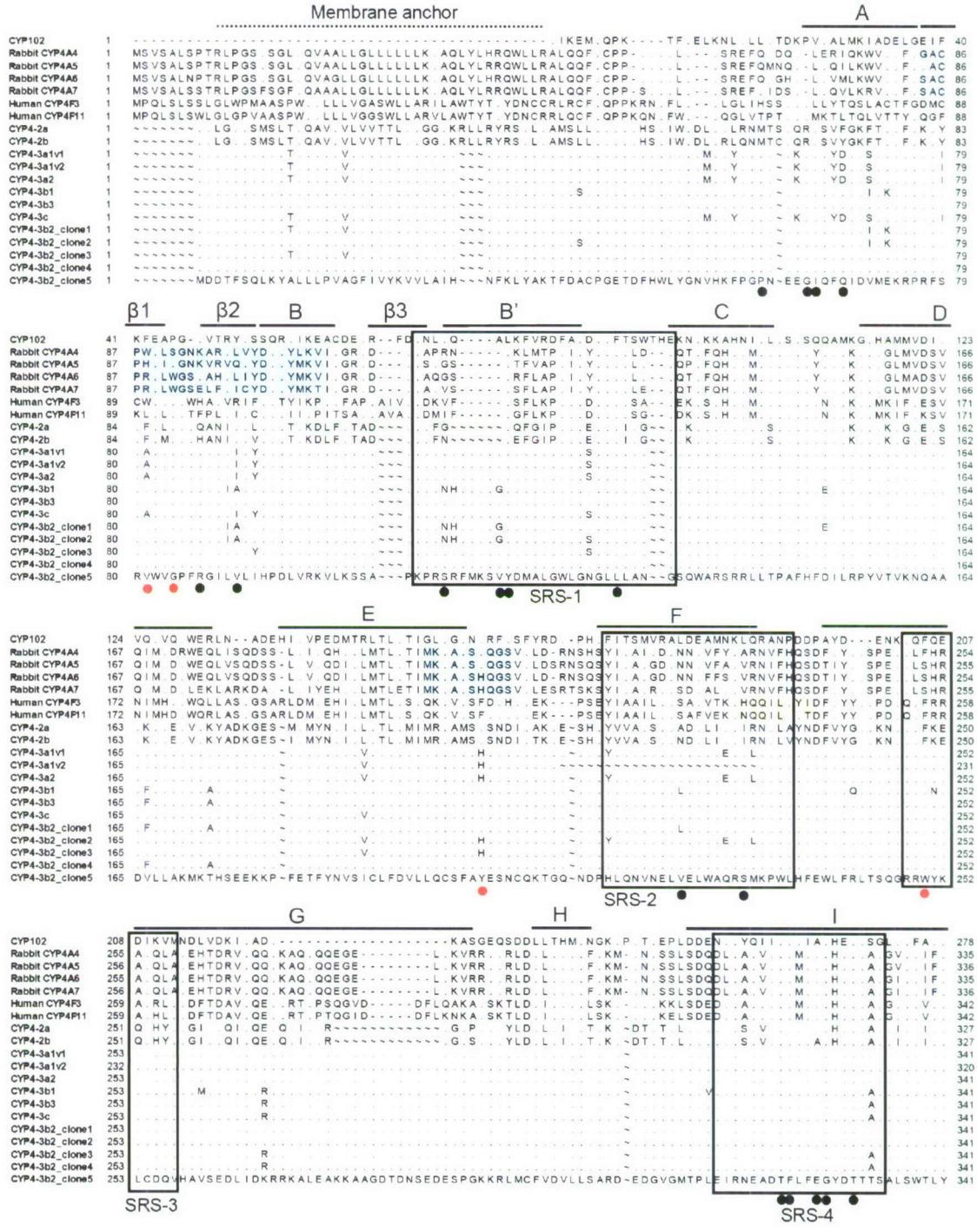
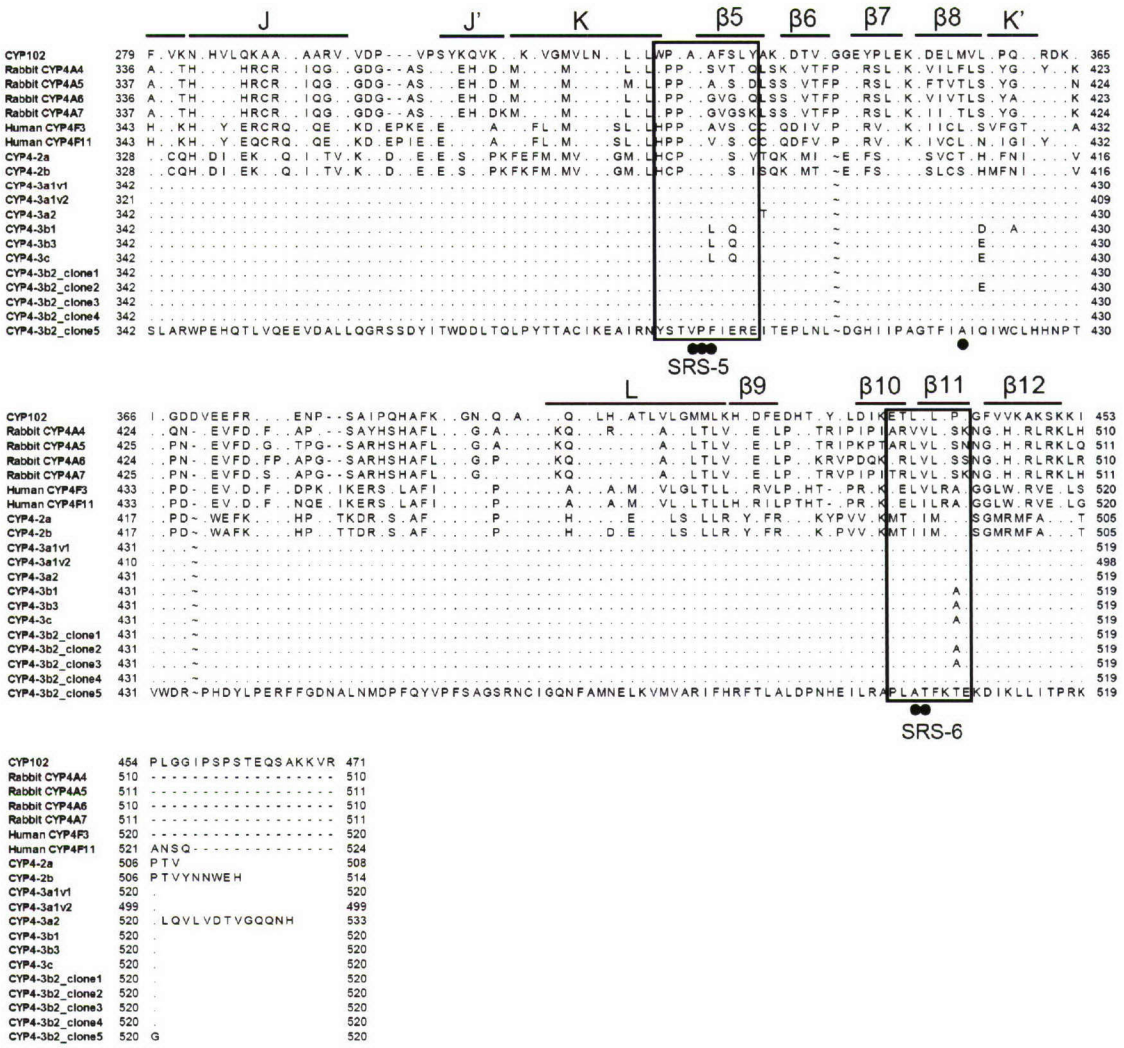


Figure 19. Comparison of CYP4 gene expression in snails feeding on control or *P. homomalla* diet for 4 d or greater. Bars represent the mean CYP4 gene expression (± SE) of snails feeding on control diet (n = 27 snails; e.g. alginic acid + squid powder) or *P. homomalla* (n = 9 snails) for 4d. Results shown here only include snails from four reefs (Big Point, North Normans, Rainbow Gardens, and Sugar Blue Holes). Run in parallel to the four day feeding assays was a series of long term feeding assays used to examine the induction of CYP4 in *C. gibbosum*. Adult *C. gibbosum* collected from North Normans were allowed to feed on control (n = 2) or *P. homomalla* (n = 1) for 14 d and mean CYP4 gene expression (± SE) is shown for each 14 d diet group . Real-time quantitative PCR was performed and the number of molecules per 0.2 μg of polyA+ RNA was calculated from the standard curve and normalized by a β-actin correction factor. Bars in the [ ] indicate molecule numbers for a subset of genes within group CYP4-3.

Figure 20. Amino acid alignment for defining putative residues involved in substrate recognition and catalytic activity in allelochemically-responsive *Cyphoma* CYP4-2 and CYP4-3 deduced amino acid sequences. In order to identify key amino acid residues, *Cyphoma* CYP4s were aligned to selected human CYP4Fs and rabbit CYP4As, whose substrate recognition sites (SRSs) have been identified in Loughran et al. (2000) and Kalsotra et al. (2004). The crystallized bacterial CYP102 provided a structural template and secondary structural elements (helices A-L and β-sheets 1-12) described in Ravichandran et al. (1993) were copied onto this alignment. Putative SRSs were also identified by aligning *Cyphoma* CYP4 deduced amino acid sequences with mammalian CYP2s from Gotoh (1992) (CYP2 sequences not included here). Residues identical to CYP4-3b2_clone 5 are indicated by a dot within the alignment. Boxed regions represent the six SRSs. Residues shaded in grey are identical in all 20 CYP sequences. The N-terminal membrane anchor region is indicated by a dotted line above the alignment. Residues lining the substrate binding channel as described in Chang and Lowe (1999) are indicated by a black dot below the alignment. Residues affecting fatty acid hydroxylase activity in rabbit CYP4A isoforms as described in Loughran et al. (2000) are indicated by a red dot below the alignment. The blue shaded boxes indicate SRS-1 and SRS-2 in rabbit CYP4A sequences defined by Loughran et al. (2000). The yellow shaded box indicates SRS-2 in human CYP4F sequences defined by Kalsotra et al. (2004). Gaps within the alignment are indicated by (~) and (—). Protein sequences used in the alignment include: bacterial CYP102 (2HPD), CYP4A4 (rabbit, P10611), CYP4A5 (rabbit, P14579), CYP4A6 (rabbit, P14580), CYP4A7 (rabbit, P14581), CYP4F3 (human, Q08477), CYP4F11 (human, Q9HBI6).


Membrane anchor
A
β1
β2
B
β3
B'
C
D
E
F
G
H
I
SRS-1
SRS-2
SRS-3
SRS-4


J
J'
K
β5
β6
β7
β8
K'
SRS-5
L
β9
β10
β11
β12
SRS-6

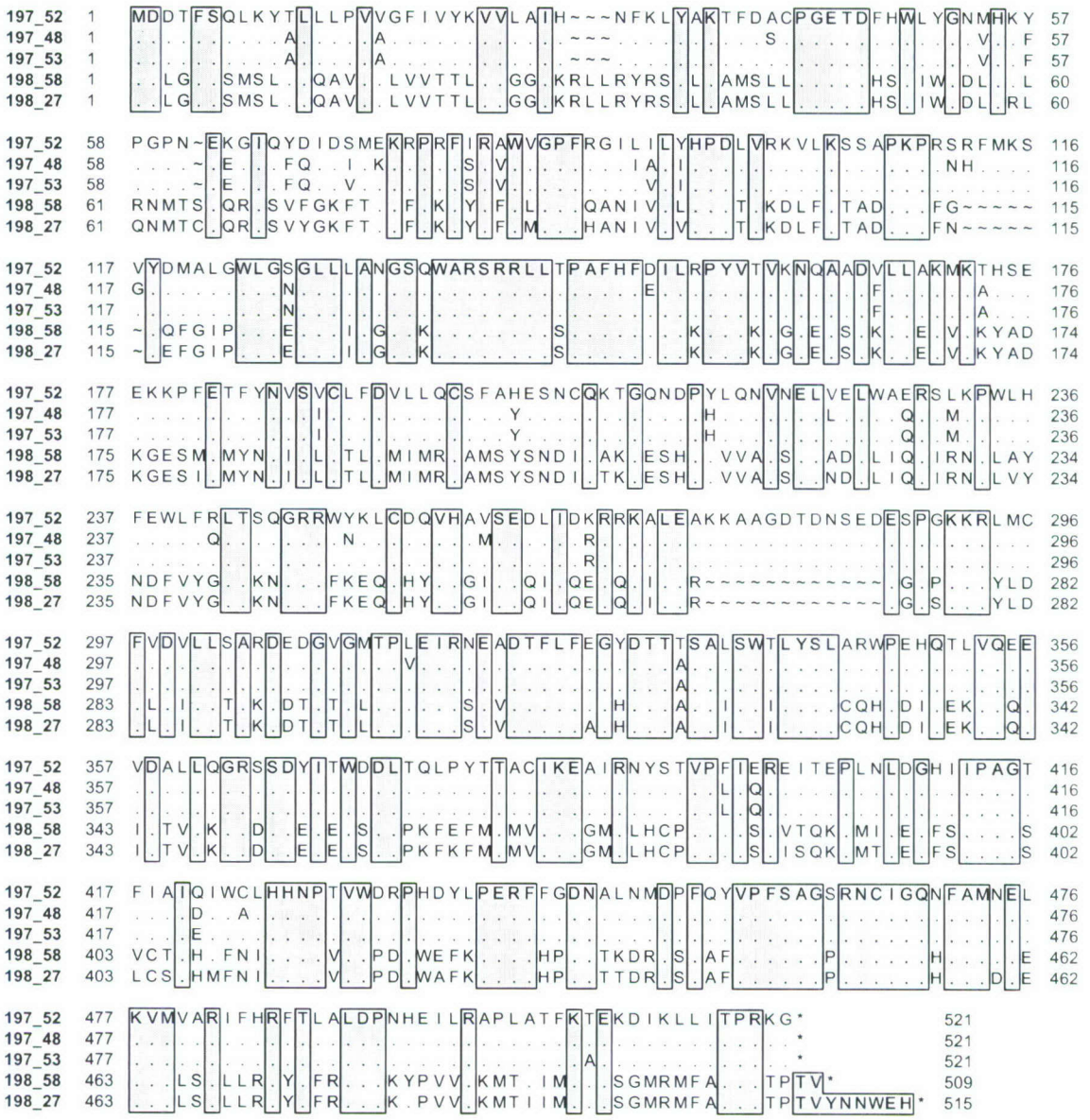


Figure 21. *Cyphoma* CYP4-2 and CYP4-3 clones selected for heterologous expression in yeast. CYP4-2a and CYP4-2b are represented by clones 198_58 and 198_27, respectively. CYP4-3a1, CYP4-3b1, CYP4-3b3 are represented by clones 197_52, 197_48, and 197_53, respectively. Deduced amino acid sequences were aligned using ClustalX. Grey boxes indicate areas of identical sequence among all clones. Amino acid residues identical to clone 197_52 are indicated by a "•". Stop codons and gaps are indicated by "*" and "~", respectively.

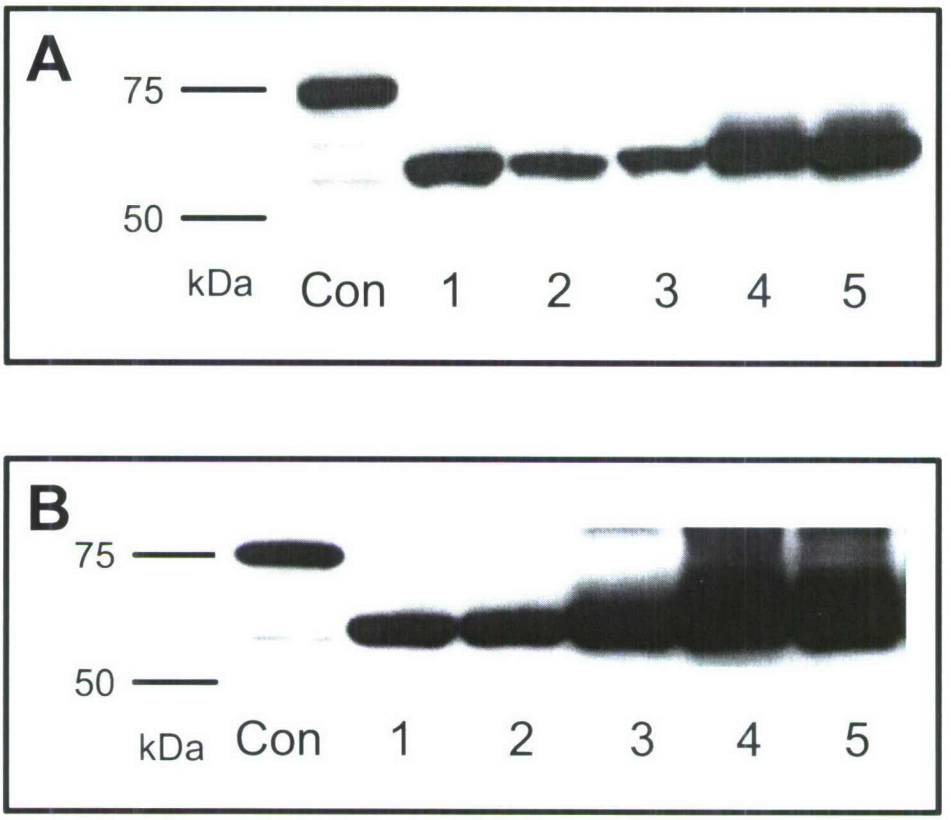
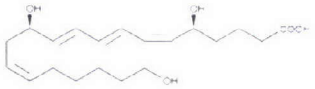


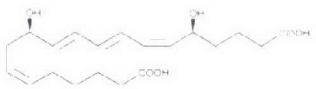
Figure 22. Recombinant expression of selected *Cyphoma gibbosum* CYPs in *Saccharomyces cerevisiae*. The W(R) strain of yeast that over-expresses NADPH-reductase was transformed with *C. gibbosum* CYPs under the control of a galactose inducible promoter. **(A)** After 8 hours **(B)** or 15 hours of induction in YPGE media containing 2% galactose, CYP expression is visualized by Western blotting with anti-V5-HRP. Each lane contains 20 μg of protein. (*lanes* – Con, positive control gene expression (*Arabidopsis* β-glucuronidase); lane 1, 198_58; lane 2, 198_27; lane 3, 197_52; lane 4, 197_48; lane 5, 197-53).

Figure 23. High pressure liquid chromatograms of leukotriene B_4 metabolites formed by recombinant yeast expressing *C. gibbosum* CYP4 proteins. Structure of metabolites formed in LTB4 hydroxylase assays are shown in (**A**). Human liver microsomes (HLM) (0.5 mg/mL protein) (**B**) and yeast microsomes (2.0 mg/mL) containing *C. gibbosum* CYP4 proteins (**C**) and were incubated with 29.7 μM LTB_4, 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride in 100 mM KPO_4 buffer at 23°C and 30°C for 120 min. Substrate and metabolites analyzed by reverse phase HPLC and separated initially with 30% acetonitrile with 1 mM perchloric acid in water changing to 70% methanol over 20 min (flow rate = 1.0 mL/min). LTB_4 hydroxylase activity was detected with recombinant *C. gibbosum* microsomes (**D**), but not in three control incubations (i.e., no protein control incubation; a 'global control' pooled yeast sample incubation without NADPH cofactor or without incubation = time zero (**E**)).

(A)

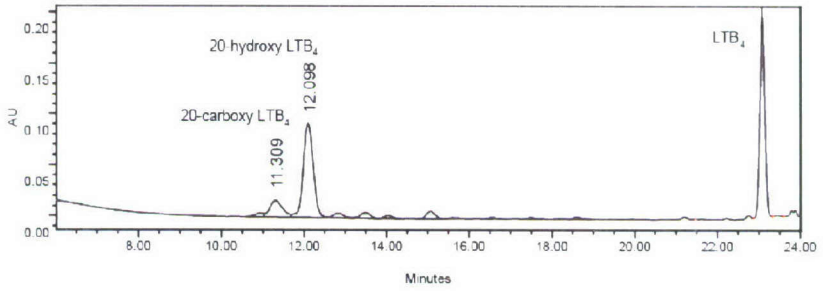


20-hydroxy LTB_4

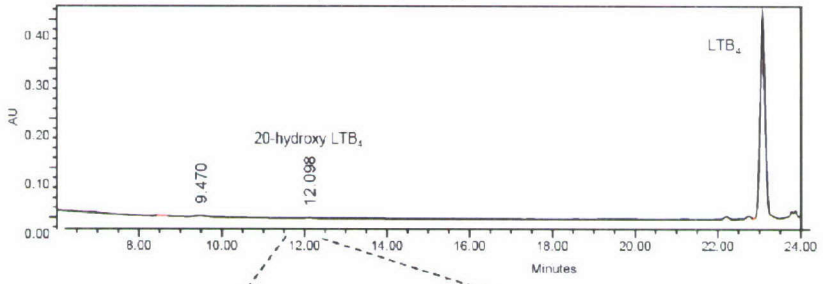


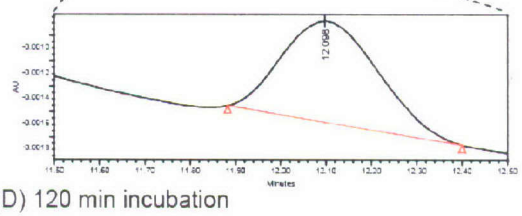
20-carboxy LTB_4

(B) Human Liver Microsomes

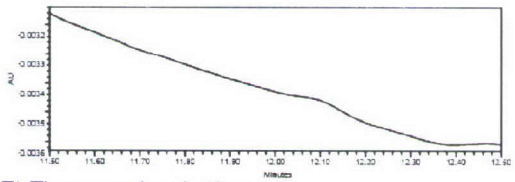


(C) Recombiant yeast expressing 197_53





(D) 120 min incubation



(E) Time zero incubation

Chapter IV

Influence of gorgonian diet on the activity and expression of *Cyphoma gibbosum* glutathione *S*-transferases (GSTs): evidence that ecologically relevant prostaglandins are substrates for molluscan GSTs.

Abstract

Despite the profound variation in tolerance of allelochemically-rich foods among marine consumers, few studies have addressed the proximate mechanisms underlying this variation. Under continual exposure to naturally occurring prey toxins, associated predators have likely evolved glutathione *S*-transferases capable of metabolizing and/or sequestering a wide range of structurally diverse compounds. GSTs were isolated from the digestive gland of the obligate gorgonian predator, *Cyphoma gibbosum*, and were studied for their sensitivity to inhibition by gorgonian extracts and commercially available prostaglandins that resemble known gorgonian allelochemicals. All gorgonian extracts examined were potent inhibitors of GST activity, suggesting that the diet of *C. gibbosum* may contain high concentrations of GST substrates. In particular, the chloroform-soluble extract from *Plexaura homomalla* substantially inhibited *Cyphoma* GST activity and was found to primarily contain prostaglandin A_2, a known gorgonian feeding deterrent. Further screening of commercially available prostaglandins in series A, E, and F revealed those prostaglandins most abundant in gorgonian tissues with highly reactive α,β-unsaturated carbonyls (PGA_2) were the most potent inhibitors. Kinetic studies of GST inhibition showed that PGA_2 was a competitive inhibitor of 1-chloro-2,4-dinitrobenzene (CDNB) conjugation by *Cyphoma* GSTs, suggesting that PGA_2 is a substrate for *Cyphoma* mu-class GSTs. *In vivo* estimates of PGA_2 concentration in digestive gland tissues calculated from snail grazing rates suggest that *Cyphoma* GSTs would be saturated with respect to PGA_2 and operating at or near physiological capacity. The high concentrations of putative substrates and inhibitors of GSTs present in all

gorgonian extracts may explain why *Cyphoma* digestive gland GSTs are highly expressed constitutively, i.e., regardless of the nature of the allelochemical diet. It is likely that GSTs operate as 'all-purpose' detoxification enzymes in this marine generalist, capable of conjugating or sequestering a broad range of gorgonian allelochemicals, potentially providing a competitive dietary advantage for this species.

Introduction

Glutathione S-transferases (GSTs, EC 2.5.1.18) comprise a large superfamily of enzymes whose soluble members function primarily as detoxification enzymes, facilitating the conjugation of a diverse array of hydrophobic electrophilic xenobiotics by the nucelophilic attack of glutathione (Parkinson 2001). GSTs have long been known as important components of cellular defense mechanisms in mammalian systems (Hayes et al. 2005) and now numerous studies are revealing their significance in mediating allelochemical tolerance in invertebrate-host interactions (reviewed in Li et al. 2007).

In terrestrial herbivores, both the diversity of GST forms and their allelochemical detoxification capabilities is likely a result of the co-evolution of these enzymes in response to host-plant allelochemicals (Wadleigh & Yu 1988, Bolton & Ahokas 1997). GST forms from polyphagous herbivores are able to metabolize a broader range of allelochemicals in comparison to those from specialists, consistent with the host range differences of these herbivores (Wadleigh & Yu 1987, 1988, Yu & Abo-Elghar 2000), suggesting that the evolution of generalist GST forms favors promiscuous catalytic activity presumably needed to cope with the breadth of dietary toxins encountered.

Additionally, the degree of polyphagy has also been correlated with the number of expressed GST isoforms. GST isoforms purified from five lepidopterans revealed that specialists expressed only one major isoform, while generalists expressed multiple forms (Yu 1989). In addition to the increased diversity and relaxed substrate specificity of generalist detoxification enzymes, the inducibility of GST forms and activity in insects exposed to allelochemicals and host plant foliage suggests that some consumers also have the ability to mount a defense against dietary toxins through changes in GST expression (Yu 1982, 1989, Brattsten 1992, Snyder et al. 1995, Yu 1999, Feng et al. 2001, Francis et al. 2001, Fang et al. 2005, Francis et al. 2005). For generalist consumers that regularly encounter unpredictable host chemistry, a combination of diverse, catalytically versatile, allelochemically-responsive GSTs would confer a selective advantage and have substantial influence in determining dietary preference among herbivores.

Like their terrestrial counterparts, marine consumers that regularly feed on allelochemically-rich prey may have evolved a parallel suite of biochemical resistance mechanisms (reviewed in Sotka & Whalen 2008). The induction or high constitutive activity of GSTs seen in several marine molluscs after exposure to allelochemical treatment or diet has been suggested as a protective mechanism against dietary intoxication (Lee et al. 1988, Vrolijk & Targett 1992, Regoli et al. 1997, DeBusk et al. 2000, Kuhajek & Schlenk 2003, Vasconcelos et al. 2007). For example, the generalist marine gastropod *Cyphoma gibbosum*, which feeds entirely on a diet of chemically-defended gorgonians (O'Neal & Pawlik 2002), contains highly expressed cytosolic GST forms in its digestive gland (Vrolijk & Targett 1992), with activities rivaling those of

terrestrial invertebrates that feed solely on allelochemically-rich prey (Lee 1991, Lee & Berenbaum 1992). Vrolijk & Targett (1992) noted differences in GST activity from field-collected *C. gibbosum* feeding on different gorgonian diets, suggesting that GST activity is induced in response to different suites of gorgonian allelochemicals.

The gorgonian *Plexaura homomalla*, in particular, has been suggested to be a preferred diet of *C. gibbosum* (Harvell & Suchanek 1987, Lasker et al. 1988) even though this gorgonian contains impressive quantities of the cyclopentenone prostaglandins (PGA_2) (Weinheimer & Spraggins 1969, Schneider et al. 1972, Spraggins 1972, Schneider et al. 1977, Ciereszko et al. 1985), known to serve as a feeding deterrent against generalist reef predators (Gerhart 1984, Pawlik & Fenical 1989, Gerhart 1991). Aside from their icythodetterent properties in marine systems, the cytotoxic effects of cyclopentenone prostaglandins (cyPGs) (e.g., induction of apoptosis and inhibition of cell growth) are believed to stem from their reactive α,β-unsaturated carbonyl group in the cyclopentenone ring, which can undergo nucleophilic addition with electrophilic moieties resulting in protein and DNA adduct formation (Noyori & Suzuki 1993, Bogaards et al. 1997, Chen et al. 1999, Sanchez-Gomez et al. 2007). Cyclopentenone prostaglandins of the A and J series have been shown to be inducers of GST activity and mRNA/protein expression in mammalian (Kawamoto et al. 2000, Uchida 2000) and invertebrate cell lines (Stanley et al. 2008). Furthermore, select vertebrate alpha-, mu- and pi-class GSTs were found to enhance PGA_2 conjugation with glutathione, suggesting that the overexpression of GST forms could modulate the cytotoxic effects of cyPGs (Bogaards et al. 1997). Given that *C. gibbosum* neither avoids *P. homomalla* nor adjusts its feeding

rates to reduce toxin exposure (Lasker et al. 1988), this snail likely possesses effective detoxification mechanisms, possibility GST-mediated, to contend with the high concentrations of dietary prostaglandins.

In a previous study, proteomic analysis confirmed the expression of two major GST mu-class isoforms responsible for the high GST activity observed in the digestive gland of *C. gibbosum* (Chapter 2). Here, I present the results of a controlled laboratory feeding study that was used to examine the influence of gorgonian diet on GST activity and subunit expression in *C. gibbosum* digestive gland. Results indicate that GST subunit composition was invariant and activity was consistently high regardless of snail diet, suggesting that constitutive GST expression in *C. gibbosum* may afford this consumer greater protection if gorgonian diets examined were to contain GST substrates. To identify possible substrates for *Cyphoma* GSTs, gorgonian extracts were screened using a bioassay-guided fractionation approach. These studies revealed that moderately hydrophobic fractions from all gorgonians examined inhibited GST activity, suggesting that the putative substrates/inhibitors for *Cyphoma* GSTs are ubiquitous among all gorgonian species. In addition, selected prostaglandins representing a range of eicosanoids previously described from *P. homomalla* were examined for their ability to inhibit GST activity. Kinetic analyses revealed cyclopentenone prostaglandins to be high affinity competitive inhibitors – and thus likely substrates – for *C. gibbosum* GSTs, with apparent affinity values comparable to K_m values described for prostaglandin-conjugating vertebrate GSTs.

Materials and Methods

Reagents

1-chloro-2,4-dinitrobenzene (CDNB), dithiothreitol (DTT), potassium phosphate, potassium chloride, EDTA, protease inhibitor cocktail mix, glutathione (GSH), and GSH-agarose were purchased from Sigma (St. Louis, MO). PD-10 desalting columns were purchased from GE Healthcare (Piscataway, NJ). Bradford reagents were purchased from BioRad (Hercules, CA). Prostaglandins 15(S)-PGA_2, 15(R)-15-methyl PGA_2, 15(S)-PGE_2, 15(R)-PGE_2, 15(S)-PGE_2-methyl ester, 15(S)-$PGF_{2\alpha}$, 15(R)-$PGF_{2\alpha}$, 15(S)-$PGF_{2\alpha}$-methyl ester were purchased from Cayman Chemical (Ann Arbor, MI).

Animal collection and feeding assay design

Feeding assays with adult *Cyphoma gibbosum* (ca 2-3 cm length) and six species of gorgonian (*Briareum asbestinum, Eunicea mammosa, Gorgonia ventalina, Pseudopterogorgia acerosa, Pseudopterogorgia americana, Plexaura homomalla*) were performed as described in Chapter 3, Materials and Methods. Briefly, a total of 39 snails were collected from four shallow reefs (Big Point – 23°47.383'N, 76°8.113'W; North Normans – 23°47.383'N, 76°8.264'W; Rainbow Gardens – 23°47.792'N, 76°8.787'W; Shark Rock – 23°45.075'N, 76°7.475'W; Sugar Blue Holes – 23°41.910'N, 76°0.23'W) surrounding Lee Stocking Island, Exuma Cays, Bahamas and allowed to feed ad. libum on either a control diet or one of six gorgonian diets for 4 d. Following the completion of the feeding assay digestive glands were immediately dissected, weighed, frozen in liquid nitrogen and maintained at -80°C until further processing. Table 1 and Figure 3 in

Chapter 3 provide a summary of the feeding assay design and summary of the digestive gland samples collected for protein analysis.

GST purification and HPLC analysis subunits

Cytosolic and affinity-purified GSTs were isolated from *Cyphoma* digestive gland samples as described in Chapter 2, Materials and Methods. Briefly, cytosolic GSTs were isolated by homogenizing digestive glands (n = 39) separately in buffer (0.1 M potassium phosphate, 1 mM EDTA, 1 mM DTT, 1.15% potassium chloride, protease inhibitor cocktail (1X); pH 7.5) and differentially centrifuging the homogenates to obtain the cytosolic fraction containing the soluble GST pool. Individual cytosolic GST fractions were then applied to both a PD-10 size exclusion and GSH-agarose affinity column in series to obtain affinity-purified GSTs as described in Chapter 2, Materials and Methods. GST fractions were buffer exchanged to low salt concentration and concentrated with Amicon Ultra-4 centrifugational filters (5K NMWL membrane; Millipore, Billerica, MA). Protein concentrations of Amicon concentrates were determined using the NanoOrange protein quantitation kit (Molecular Probes, Eugene, OR).

A 30 µL aliquot of the affinity-purified GST concentrate from each of the 39 digestive gland samples was injected onto a reverse phase Vydac protein/peptide column (model #218 TP 52; C18 µm 250 mm x 2.1 mm) and separated using a Waters 600 MultiSolvent Delivery System, with a flow rate of 0.5 mL/min. Peaks were detected using a Waters 2487 Dual Wavelength Absorbance Detector (λ = 214 nm). Mobile phase A consisted of 38% acetonitrile, 62% water and 0.1% trifluoroacetic acid (TFA). Mobile

phase B consisted of 80% acetonitrile, 20% water and 0.1% TFA. The initial mobile phase consisted of 100% A. GST subunits were separated using a linear gradient from 0 to 40% B in 22 min, and 40 to 100% B in 37 min. The column was re-equilibrated with 100% A from 37-50 mins prior to the next injection. Integration of peak area was achieved using the Empower 2 Chromatography Data Software package (Waters, Milford, MA) and converted to GST subunit percent composition for each digestive gland sample.

GST activity assay

Enzyme activity was measured using CDNB as a substrate by the method of (Habig et al. 1974) optimized for *C. gibbosum* (Vrolijk & Targett 1992) in a microplate format. The reaction mixture (in a final volume of 200 µL) contained 0.1 M potassium phosphate buffer, 1.0 mM EDTA, pH 7.5, 1 mM CDNB, 1 mM reduced glutathione (GSH) and 2 µg of cytosolic protein or 3.3 – 6.4 ng of affinity-purified GST sample. CDNB was solubilized in ethanol and constituted 1% of the final reaction mixture volume. The reaction was incubated at 25°C and initiated by the addition of CDNB. All reactions were performed in triplicate. The conjugation of CDNB with GSH was measured as the increase in absorbance at 340 nm ($\Delta\varepsilon_{340}$ 0.00503 μM^{-1} cm^{-1}) using a tunable microplate reader (Versamax, Molecular Devices, Sunnyvale, CA). Activity was calculated using protein concentrations determined via the Bradford assay with BSA as a standard.

Extraction and isolation of gorgonian compounds

A minimum of ten colonies for each gorgonian species were collected from shallow reefs (< 20 m) surrounding Lee Stocking Island, Exuma Cays, Bahamas and used in the feeding assays described above. A portion of the gorgonian colonies, prior to their introduction into the feeding assay, was immediately removed after field collection and immersed in seawater to determine volumetric displacement, frozen at -80°C, and lyophilized for subsequent chemical extraction. *Pseudopterogoria blanquillensis* was also collected for chemical analysis; however, this gorgonian species did not participate in the feeding assays. A 50 mL equivalent pool of gorgonian tissue for each of the eight species was extracted twice in 250 mL reagent grade acetone overnight with agitation. Resulting extracts were vacuum-filtered through celite, dried down by rotary evaporation, and recombined into a 20 mL scintillation vial using a minimum volume of solvent. Extracts were then completely dried using a vacuum concentrator. The crude extracts were assayed at $1/20^{th}$ natural concentration by volume (i.e., the extract from 0.05 mL of gorgonian was diluted into 1 mL of assay buffer) for the ability to inhibit *Cyphoma* cytosolic GST activity as described above, and inhibitory fractions were subjected to further fractionation using a bioassay-guided fractionation approach.

Gorgonian crude organic extracts were separated by partition between hexane and methanol-water (9:1) followed by partition of the methanol-water fraction (adjusted to 6:4) against chloroform. All three fractions were reduced *in vacuo* and assayed for their ability to inhibit cytosolic GST activity. The chloroform fractions from all eight gorgonian species showed the most inhibition and were further separated on a reverse-

phase semi-prep Zorbax SB-C18 column (5 μm, 9.4 mm x 2.5 cm) attached to a Waters Breeze HPLC system (515 pump) with a Waters 2487 UV detector at 215 and 254 nm. Compounds were eluted over 33 mins at a flow rate of 3 mL/min with methanol/water (9:1) with linear ramping to 100% methanol. HPLC fractions were hand collected at three minute intervals over 33 min, yielding ten fractions per gorgonian species. Each fraction was assayed for GST inhibition at $1/10^{th}$ natural concentration by volume. Fractions yielding 100% inhibition were further assayed at $1/200^{th}$ natural volumetric concentrations.

Inhibition assays

GST activity measurements were performed as described above. The reaction mixture (in a final volume of 200 μL) contained 0.1 M potassium phosphate buffer, 1.0 mM EDTA, pH 7.5, 1 mM CDNB, 1mM reduced GSH and either 2 μg of cytosolic GST preparations or 3.3 – 6.4 ng of affinity-purified GSTs. Gorgonian extracts were dissolved in appropriate solvents (e.g., acetone, *n*-propanol, or methanol), HPLC fractions were dissolved in methanol, and prostaglandins were dissolved in DMSO. Solvent concentrations did not exceed 5% of the experimental volume and had no effect on GST activity when compared to non-solvent controls (data not shown). Immediately prior to the start of the assay, inhibitor solutions were added to the buffer/GSH mixture and homogenized to ensure equal distribution of inhibitor in all microplate wells. The data were corrected for the non-enzymatic reaction rates and the effect of the inhibitors on

catalytic activity was measured by comparing the initial rate of reaction in the presence and absence of the inhibitor.

Initially, eight prostaglandins (15(S)-PGA_2, 15(R)-15-methyl PGA_2, 15(S)-PGE_2, 15(R)-PGE_2, 15(S)-PGE_2-methyl ester, 15(S)-$PGF_{2\alpha}$, 15(R)-$PGF_{2\alpha}$, 15(S)-$PGF_{2\alpha}$-methyl ester) were screened at 600 μM for their potential to inhibit cytosolic GST activity. From this initial screening, only those prostaglandin compounds that demonstrated an ability to reduce GST activity by 50% or greater were further evaluated at a range of concentrations (0.2 – 2000 μM) in order to estimate the concentration producing 50% inhibition of enzyme activity (IC_{50}). Prostaglandin IC_{50} values were calculated and 95% confidence intervals were estimated using Prism 5.0 software (GraphPad) by fitting the log transformation of the response variable by nonlinear regression to the variable slope equation (1) and constraining the bottom to zero while allowing the Hill Slope to vary. The variable slope equation is:

$$Y = \frac{\text{Bottom} + (\text{Top} - \text{Bottom})}{1 + 10^{((\text{Log}IC_{50} - [I])^* \text{HillSlope})}} \qquad (1)$$

where Top is the maximum percent GST activity remaining, Bottom was constrained to zero, IC_{50} is the concentration of inhibitor that produces inhibition half-way between the Top and Bottom, and [I] is the logarithmic concentration of the inhibitor.

Determining inhibition type and kinetic constants

In order to determine the type of inhibition, initial-rate measurements with 15(S)-PGA_2 and GSH using CDNB as the concentration-variable substrate were performed with

an affinity-purified GST sample at 25°C in 0.1 M potassium phosphate buffer, 1.0 mM EDTA, pH 7.5, containing 4% (v/v) DMSO. Data were corrected for the non-enzymatic reaction rates and globally fitted to equations for competitive (Eq. (2)), noncompetitive (Eq. (3)), and uncompetitive (Eq. (4)) using a single substrate-single inhibitor model with Prism 5.0 software. The equations used were:

$$v_o = \frac{V_{max}[S]}{K_m(1 + [I]/K_i) + [S]} \quad (2)$$

$$v_o = \frac{V_{max}[S]}{K_m(1 + [I]/K_i) + [S](1 + [I]/K_i')} \quad (3)$$

$$v_o = \frac{V_{max}[S]}{K_m + [S](1 + [I]/K_i')} \quad (4)$$

where v is the initial rate of product formation, V_{max} is the maximal rate of product formation, K_m is the Michaelis-Menten constant, [S] is the substrate concentration, [I] is the inhibitor concentration, K_i is the equilibrium dissociation constant for the enzyme-inhibitor complex, and K_i' is the equilibrium dissociation constant for the inhibitor from the enzyme-substrate-inhibitor complex.

The appropriate model of inhibition was chosen based on how well the data supported each model (goodness-of-fit), given the number of parameters and sample size, using both the Akaike information criterion (AIC) (Akaike 1986) and the extra-sum-of-squares (*F*-test) tests. In paired model comparisons (i.e., Eq. (2) versus Eq. (3)) performed by Prism 5.0, the resulting ΔAIC_c values were used to calculate the probability

that each model was correct given the available data and AIC results were compared to *F*-test results to select the most appropriate inhibition model. In no case did the two test results (Akaike and extra-sum-of-squares) differ from one another. The data were then fitted to the most appropriate model and used to estimate an apparent Michaelis-Menten constant (K_m) and maximal velocity (V_{max}). Double reciprocal plots of the inhibition data were used to examine the inhibition patterns.

Because the IC_{50} depends on the substrate concentration used in the experiment, this value is only useful for comparing inhibitors within experiments and not between laboratories unless identical assay conditions were used. However, calculated K_i values (the dissociation constant of the enzyme-inhibitor complex) can be used to directly compare inhibitor affinity for the enzyme between studies. Once 15(S)-PGA_2 was confirmed to be a competitive inhibitor of *C. gibbosum* GST activity, the K_i was calculated using the IC_{50} values obtained for 15(S)-PGA_2, 15(R)-15-methyl PGA_2, 15(S)-PGE_2, 15(S)-$PGF_{2\alpha}$ with the Cheng-Prusoff equation (Eq. 5), where IC_{50}, K_m, S, and K_i are defined above.

$$IC_{50} = K_i \left(1 + \frac{S}{K_m}\right) \qquad (5)$$

Results

Dietary influence on GST activity and protein composition

Cytosol was isolated from the digestive glands of 39 individual *C. gibbosum* feeding on one of six gorgonian diets (n = 27) or a control diet (n = 12) for four days. GST specific activity levels measured from individual crude cytosolic preparations were

grouped by snail diet and ranged from 1930 to 2957 nmol min^{-1}mg $protein^{-1}$. GST activity values were within the range reported by Vrolijk and Targett (1992) but did not differ significantly between snail diets (Figure 1). Cytosolic GSTs were further purified (approximately 200-fold) by size-exclusion and affinity chromatography (see Chapter 2, Results) and separated by reverse-phase HPLC in order to quantify the relative abundance of GST subunits from each digestive gland sample (Figure 2). HPLC separation identified fourteen unique peaks, with two major peaks (4 and 8) representing on average 25% and 68% of the relative composition of GST subunits among all digestive gland samples, respectively (Figure 3). HPLC peak (1) and peaks (2 – 14) were previously identified as theta- and mu-class GSTs, respectively (Chapter 2, Results). The relative amounts of each GST form did not differ significantly as a function of gorgonian diet when expressed either as a percent of all subunits present (Figure 3) or when normalized to the amount of affinity-purified GST sample injected on to the HPLC column (Figure 4). These results indicate that, while GST activity is constitutively expressed at high levels in *Cyphoma* digestive gland, both GST activity and subunit abundance are unaffected by gorgonian diet.

Inhibition of GST activity by gorgonian extracts

Crude organic extracts were prepared from eight species of gorgonian (*B. asbestinum, E. mammosa, G. ventalina, P. acerosa, P. americana, P. blanquillensis, P. elisabethae,* and *P. homomalla*) and tested at $1/20^{th}$ natural volumetric concentrations for inhibition of *Cyphoma* GST activity (Figure 5). Extracts from both *P. acerosa* and *P.*

homomalla completely inhibited GST activity, while extracts from the remaining gorgonian species inhibited ≥ 70% of GST activity compared to solvent controls. Crude extracts were then partitioned into three fractions (e.g., hexane, chloroform, and aqueous) of increasing polarity. The chloroform soluble fractions from all gorgonian species consistently showed ≥ 80% inhibition of GST activity compared to controls. Aqueous fractions displayed minor inhibitory effects with the exception of fractions from *P. acerosa* and *P. homomalla,* which inhibited GST activity by 85 and 99%, respectively. Hexane fractions exhibited intermediate and wide-ranging inhibitory effects depending on gorgonian species. It was decided to further separate the chloroform soluble fraction by HPLC because of the consistency of inhibition seen for this fraction among all gorgonian extracts surveyed.

Chloroform soluble fractions were separated using reverse-phase HPLC. For all gorgonian species examined, compounds eluting between 3 to 6 min (fraction 1) consistently inhibited affinity-purified GST activity by > 80% at $1/10^{th}$ natural volumetric concentrations compared to paired solvent controls (Figure 6). Inspection of HPLC traces suggests that fraction 1 for all gorgonian species examined consists of a mixture of compounds. HPLC fraction 1 was further diluted to $1/200^{th}$ natural volumetric concentrations for those gorgonian species (*B. asbestinum, E. mammosa, P. acerosa, P. homomalla*) exhibiting > 93% inhibition of GST activity. Diluting fraction 1 decreased the inhibitory effect of the compound(s); however, in all cases diluted fractions still retained the ability to inhibit > 65% GST activity compared to solvent controls.

The highly inhibitory chloroform fraction 1 from *P. homomalla* was further analyzed by Amy Lane at the Georgia Institute of Technology by high-performance separation techniques coupled with spectroscopic methods such as mass spectrometry (MS) and nuclear magnetic resonance (NMR) to provide additional structurally informative data. LC-MS analysis of *P. homomalla* chloroform-soluble fraction 1 revealed the presence of three peaks (m/z = 271, 315, 333), which are diagnostic of the free hydroxy acid of PGA_2 (Figure 7). Further comparison of the ^1H NMR spectra of *P. homomalla* (fraction 1) and the PGA_2 standard (Figure 8) confirmed the presence of PGA_2 in the gorgonian tissue and indicated that the free hydroxy acid of PGA_2 was a major component of the chloroform-soluble fraction. A dilution series of the PGA_2 standard was subjected to LC-MS analysis and used to quantify the amount of PGA_2 in *P. homomalla* tissue (1.6 mM) based on an extrapolation from the standard curve. Further analysis of *P. homomalla* HPLC fractions 1 and 2 by LC-MS and NMR also suggested the presence of additional prostaglandins, possibly PGA_2 analogs (data not shown).

Inhibition of GST activity by prostaglandins

Eight commercially available prostaglandins representing a diversity of forms present in gorgonian tissue (i.e., PGA_2, PGE_2, $PGF_{2\alpha}$), including both enantiomers (R and S) forms when possible, were screened at 600 μM for their ability to inhibit CDNB-conjugating activity of crude cytosolic *Cyphoma* GSTs (Figure 9). Prostaglandins containing a cyclopentenone ring (PGA_2) caused the greatest inhibition of GST activity, while the methyl ester forms of PGE_2 and $PGF_{2\alpha}$ displayed little to no inhibitory activity.

In general, prostaglandin series with the greatest inhibitory activities (i.e., PGA_2) were those reported to be found in the greatest abundance in gorgonian tissues. The potencies of the four most inhibitory prostaglandins (15(S)-PGA_2, 15(R)-15-methyl PGA_2, 15(S)-PGE_2, 15(S)-$PGF_{2\alpha}$) were evaluated at a range of concentrations (0.2 – 2000 μM) (Figure 10). All displayed concentration-dependent inhibition of enzyme activities with IC_{50} values ranging from 75.4 μM for 15(S)-PGA_2 to 334.6 μM for 15(S)-$PGF_{2\alpha}$ (Table 1).

The inhibitory effect of 15(S)-PGA_2 was studied in greater detail by examining CDNB concentration-dependent GST conjugating activity in the presence of two concentrations of inhibitor. Apparent kinetic constants (K_m and V_{max}) for CDNB in the presence and absence of 15(S)-PGA_2 (Table 2) were obtained by fitting the data to the Michaelis-Menten equation by non-linear regression. V_{max} values for CDNB in the presence and absence of inhibitor did not differ significantly from one another (Extra sum-of-squares F test, Experiment 1, $F_{1,32} = 2.462$, $p = 0.13$; Experiment 2, $F_{1,31} = 2.471$, $p = 0.13$); however, precise determination of this parameter proved difficult due to the high variability in GST activity measurements at high concentrations of CDNB (Figure 11, part A, B), due to the limited solubility of this substrate at concentrations greater than 3 mM.

To determine the type of inhibition caused by PGA_2, the data were fit to three inhibition models (competitive, noncompetitive, and uncompetitive) in a series of pairwise comparisons (Table 3, 4). Both the Akaike's information criterion and the extra-sum-of-square F test were in agreement that competitive inhibition was statistically superior for describing the data. The competitive inhibition model was, therefore, used to

calculate kinetic constants for CDNB (K_m = 0.25 – 0.47 mM) and 15(S)-PGA_2 (K_i = 15.6 – 24.2 μM) (Table 5). The Cheng-Prusoff equation for competitive inhibition was also used to calculate the corresponding K_i values for 15(S)-PGA_2, 15(R)-15-methyl PGA_2, 15(S)-PGE_2, and 15(S)-$PGF_{2\alpha}$ using the IC_{50} values listed in Table 1. The average K_i value for 15(S)-PGA_2 derived from the competitive inhibition equation (K_i = 19.9 μM, Table 5) exactly matched the calculated K_i value obtained using the IC_{50} values obtained for 15(S)-PGA_2 (K_i = 19.9 μM, Table 1).

Lineweaver-Burk plots of 1/V versus 1/[CDNB] for 15(S)-PGA_2 inhibition of affinity-purified GSTs did not converge on the y-axis as expected for competitive inhibition, suggesting mixed (competitive and noncompetitive) inhibition (Figure 11, part C, D). Because inhibition assays were not performed on single GST isoform preparations but rather on affinity-purified GST fractions containing multiple GST isoforms, it is possible that PGA_2 could interact differently with select GST isoforms. In addition, the variability of the data, likely a result of the insolubility of CDNB at concentrations above 3 mM, could substantially influence the trajectory of the regression analysis. In contrast to the Lineweaver-Burke plots, the weight of evidence from the more statistically valid nonlinear regression analysis suggests that PGA_2 is a competitive inhibitor and putative substrate for *Cyphoma* GSTs.

Discussion

It is likely that the exploitation of allelochemically-defended gorgonian corals by the co-evolved predator, *Cyphoma gibbosum*, is facilitated by this predator's ability to

biotransform or sequester dietary allelochemicals using detoxification enzymes such as soluble glutathione *S*-transferases. GSTs are integral components of the cellular xenobiotic defense system (Strange et al. 2000) and have been documented to mediate allelochemical tolerance in terrestrial consumers (Wadleigh & Yu 1987, 1988, Yu & Abo-Elghar 2000). Here, I investigated the influence of gorgonian diet on the expression, activity and inhibition of *Cyphoma* GSTs.

Cyphoma cytosolic digestive gland preparations contained high GST activity; however, contrary to previous reports (Vrolijk & Targett 1992), GST activity did not significantly vary with respect to gorgonian diet. The apparent differences likely lie in the basic experimental design between the two studies. Vrolijk & Targett (1992) noted differences in GST activity among field-collected individuals where no data were available on the residence time of snails on their respective hosts. In contrast, snails in the present study were subject to controlled 4 d feeding assays. It is possible that GST enzymes could show significant levels of induction if snails were allowed to feed on gorgonian diets longer than four days. However, it seems unlikely that our design, which included those gorgonian species examined by Vrolijk & Targett (1992), would have missed significant induction of GSTs because the length of the assay should have provided sufficient time for protein induction based on GST induction studies in polyphagous insects exposed for two days to dietary allelochemicals (Wadleigh & Yu 1988, Yu 1999). Additionally, snails could extend their exposure to the same suite of allelochemicals beyond the average 3.3 day residence time predicted by (Harvell & Suchanek 1987), if snails migrated to another colony of the same species. This scenario

would be favored if the proportion of *B. asbestinum* and *G. ventalina*, the two gorgonian diets eliciting increased GST activity in Vrolijk & Targett (1992), were in higher abundance on reefs because prey selection by *C. gibbosum* is in proportion to gorgonian species abundance (Harvell & Suchanek 1987). Alternatively, geographical (Rodriguez 1995) and within colony differences in allelochemical content (Harvell & Fenical 1989) could account for the differences between the two studies.

Although GST activity did not vary by gorgonian diet, cytosolic digestive gland GSTs were further purified by affinity chromatography to investigate if GST subunit composition was influenced by allelochemical exposure. *Cyphoma* GST subunits were separated by HPLC resulting in the identification of two major mu-class GST subunits accounting for 93% of the total GST subunit abundance. Quantification of GST subunit composition indicated that the relative abundance of GST subunits did not differ among snails feeding on different gorgonian diets. Interestingly, GST activity was maintained at a high level and subunit composition did not vary in snails fed control diets devoid of allelochemicals as compared to snails fed gorgonian diets. The presence of high GST activity in control-fed snails could indicate that some lipophilic gorgonian compounds and/or their metabolites may persist in snail tissues even after feeding has ceased, causing the expression of GSTs to be maintained. Alternatively, *Cyphoma* GSTs could be expressed constitutively at high levels or regulated by an allelochemical-independent mechanism. Having a constant supply of 'all-purpose' GST enzymes may prove advantageous for predators that consistently feed on prey containing allelochemical GST substrates.

To determine the prevalence of gorgonian species containing putative GST substrates, gorgonian extracts and semi-pure fractions were screened for their ability to inhibit the CDNB-conjugating activity of *Cyphoma* GSTs. The ability of compounds to inhibit GST activity was used as an indirect measure of their potential to act as GST substrates. Crude extracts from all eight gorgonian species were effective at inhibiting GST activity by greater than 70% at $1/8^{th}$ natural volumetric concentrations compared to controls. In general, substrates for GST enzymes are classified as hydrophobic compounds able to react with the thiol moiety of glutathione (Hayes et al. 2005). Upon further separation of the crude extract, the chloroform-soluble fraction was identified as containing the bulk of the inhibitory compounds. Efforts are underway to determine if the chloroform-soluble fractions contain electrophilic compounds with internal leaving groups (e.g., epoxides, α,β-unsaturated ketones, halogenated hydrocarbons, etc.) (van Bladeren 2000) capable of serving as sites for the nucleophilic addition of glutathione.

In addition to containing potential GST substrates, gorgonian extracts may also contain electrophilic compounds that could act as potent GST inhibitors, binding to free cysteine residues on the protein, resulting in enzyme inactivation (van Iersel et al. 1999). The presence of high affinity GST inhibitors in gorgonian tissues may represent specific counter-adaptations of prey to thwart consumer GST-mediated metabolism of co-occurring allelochemicals (Lee 1991, Li et al. 2007). Although the high-throughput screening approach of gorgonian extracts was not able to distinguish between GST substrates and inhibitors, this result did substantiate the hypothesis that all gorgonian species contained significant quantities of compounds capable of interacting with

Cyphoma GSTs, which could account for the high constitutive activity of digestive gland GSTs identified here and in (Vrolijk & Targett 1992).

In this study, I also examined the chemistry of specific gorgonian diets that contain classes of compounds recognized to be substrates/inhibitors for vertebrate GSTs (Cagen et al. 1975, van Iersel et al. 1999, Beuckmann et al. 2000, Kawamoto et al. 2000, Uchida 2000, Murphy & Zarini 2002, Sanchez-Gomez et al. 2007). The gorgonian *Plexaura homomalla*, once under intense study because of the unusually high concentration of prostaglandins isolated from its tissues, can account for nearly 50% of the biomass on some Caribbean reefs (Bayer & Weinheimer 1974) and has been suggested to be a favored diet of *C. gibbosum* (Harvell & Suchanek 1987, Lasker et al. 1988, Chiappone et al. 2003). The potential importance of this particular gorgonian species' allelochemicals in the co-evolution of *C. gibbosum* detoxification enzymes coupled with the interesting ecological and biological activities of eicosanoids, were motivating factors in determining if prostaglandins could serve as substrates for *C. gibbosum* GSTs.

P. homomalla tissues predominately contain the fully esterified form of PGA_2 (~ 2% dry weight of the gorgonian) (Weinheimer & Spraggins 1969), which is related to a larger group of eicosanoids that includes the coral-derived halogenated marine clavulones (Honda et al. 1987, Iguchi et al. 1987) and puniglandins (Fukushima & Kato 1985, Baker & Scheuer 1994), all of which display cytotoxic activities thought to be related to the presence of a reactive α,β-unsaturated ketone (Honn & Marnett 1985, Kato et al. 1986). While the exact mechanism of toxicity is unknown, the prostaglandins are transported

into the nucleus (Narumiya & Fukushima 1986, Narumiya et al. 1987, Narumiya et al. 1988) where the electrophilic α,β-unsaturated carbonyl is free to bind with nucleophilic sulfhydryl residues on target proteins, unless rapidly conjugated by cytosolic GSH and exported out of the cell by glutathione-conjugate transporters (Atsmon et al. 1990). In this study, the α,β-unsaturated carbonyl-containing prostaglandins (15(R)-15-methyl PGA_2 and 15(S)-PGA_2) were the most potent inhibitors of CDNB conjugating activity of *Cyphoma* GSTs in both the initial screening of eight prostaglandin compounds and upon comparison of IC_{50} values, establishing the order of potency of prostaglandins to be 15(S)-PGA_2 > 15(R)-15-methyl PGA_2 >> 15(S)-PGE_2 ≈ 15(S)-$PGF_{2\alpha}$. The K_i values for cyclopentenone-containing prostaglandin A series were also 2.3- to 4-fold lower (greater affinities) for *Cyphoma* GSTs in comparison to those of either PGE_2 or $PGF_{2\alpha}$. In addition, subsequent structural analysis confirmed that the inhibitory *P. homomalla* chloroform-soluble fractions predominantly contained high concentrations of the potent PGA_2 and PGA_2-analogs, likely accounting for the bulk of the observed inhibition for these particular HPLC fractions.

Statistical comparison of the goodness-of-fit of the data among different models of inhibition using both Akaike information criterion and extra-sum-of-squares test suggest that PGA_2 is a competitive inhibitor of CDNB, and likely a substrate for *C. gibbosum* GST(s). Because PGA_2 acts as a competitive inhibitor of GST activity, likely binding with high affinity in the active site (H-site) once occupied by CDNB, it is reasonable to compare K_i values (i.e., apparent K_m for PGA_2) obtained here to K_m values for PGA_2 cited in other studies. The apparent K_m (~ 19.9 μM) for 15(S)-PGA_2 described

here is in line with the K_m value identified for the conversion of PGA_2 to its glutathione conjugate by human mu-class GST M1a-a (26 μM) (Bogaards et al. 1997) and K_m values for PGA_2 for rat alpha-class GST A4-4 (12 μM) and human mu-class GST M2-2 (7.6 μM) (Hubatsch et al. 2002). The similarity between kinetic constants further supports the conclusion that PGA_2 is a substrate for *Cyphoma* GSTs. The rank order of affinity of *Cyphoma* GSTs for prostaglandins (15(S)-PGA_2 > 15(R)-15-methyl PGA_2 > 15(S)-PGE_2 > 15(S)-$PGF_{2\alpha}$), also is positively correlated with the abundance of each prostaglandin series in *P. homomalla* tissues, suggesting that *Cyphoma* GSTs may have evolved to efficiently catalyze those prostaglandin series found in the greatest abundance in its diet (PGA_2), yet still retain a broad enough substrate specificity to conjugate additional forms (PGE_2, $PGF_{2\alpha}$).

The apparent K_m values reported here indicate that dietary prostaglandins would be high affinity substrates of *Cyphoma* digestive gland GSTs *in vivo*. However, the physiological relevance of GSTs for prostaglandin biotranformation depends on the concentrations of prostaglandins occurring in the digestive glands of *Cyphoma* feeding on prostaglandin-containing gorgonians. To obtain an estimate of these concentrations, I first calculated the volume of *P. homomalla* tissue consumed per snail per day based on feeding scar measurements reported in (Harvell & Suchanek 1987). *Cyphoma* feeding scars on *P. homomalla* colonies averaged 12 cm in length (n = 21 snails), did not exceed 1 cm in width, and penetrated to the gorgonian skeleton 66% of the time (Harvell & Suchanek 1987). Therefore, conservative estimates of scar volume averaged 1.44 cm^3 per snail (or 1.44 mL) (length x width x depth; 12 cm x 0.4 cm x 0.4 cm). This tissue

volume was divided by the mean residence time of snails feeding on *P. homomalla* (2.9 d, n = 50 snails) (Harvell & Suchanek 1987) to yield an estimate for the volume of *P. homomalla* tissue consumed by each snail per day (0.66 mL/snail/day). (Pawlik & Fenical 1989) determined that 1 mL of wet *P. homomalla* tissue (excluding the gorgonian axial skeleton) was equal to 0.86 g of dry gorgonian tissue. If 2% of the dry weight of the gorgonian is prostaglandins (Weinheimer & Spraggins 1969), then 0.66 mL of gorgonian tissue would contain 0.011 g of prostaglandins. Assuming the majority of the prostaglandins are in the PGA_2 (FW = 348.5 g/mol) form and are completely retained within the digestive gland upon ingestion (ave. dig. gland weight = 0.25 g (n = 96 snails) with a density comparable to human liver ~1 g/mL), then the upper limit of PGA_2 concentrations in digestive gland would be 0.13 M. The single day grazing rates of *P. homomalla* colonies by *C. gibbosum* reported in (Lasker et al. 1988) were used to obtain a lower bound of tissue consumption; their average feeding rates of 0.17 mL/snail/day lead to an estimated PGA_2 concentration of 0.03 M. Ciereszko & Schneider (1987) reported the fecal pellets of *C. gibbosum* contain no appreciable amounts of recognizable prostaglandins, suggesting that the majority of prostaglandins are being metabolized or sequestered in the snail. If we conservatively assume only 1% of the ingested prostaglandins are retained within the digestive gland during feeding (e.g., 1% concentration of PGA_2 ~ 0.3 – 1.3 mM PGA_2), the *in vivo* concentration of prostaglandins in this tissue would still be 15- to 65-fold higher than the apparent K_m (15.6 – 24.2 μM) obtained for PGA_2 (Table 5). Even at the lower bound of *in vivo* PGA_2

concentration (~ 0.3 mM), *Cyphoma* GSTs would be nearing their physiological capacity (>95%) according to fractional velocity (v/V_{max}) calculations.

The K_m for CDNB calculated here (ave. 0.36 mM) was substantially lower than the K_m value (ave. 1.49 mM) reported for *Cyphoma* by Vrolijk and Targett (1992), indicating that CDNB has a higher affinity for *Cyphoma* GSTs than previously thought. The apparent differences between kinetic constants is likely a result of measurements made using different protein sources – affinity-purified GSTs used in this study as opposed to crude cytosolic preparations used in Vrolijk and Targett (1992). If CDNB readily interacts with non-GST proteins in a sample, effectively decreasing the free-CDNB concentration, this could result in higher apparent K_m values reported by Vrolijk and Targett (1992).

GSTs have gained considerable attention for their conjugating ability to conjugate electrophilic toxicants. However, their lesser known ability to bind non-substrate ligands by acting as 'xenobiotic sponges', capable of soaking up and sequestering excess intracellular xenobiotics, may be as important of a protective mechanism (Ketley et al. 1975, Kostaropoulos et al. 2001). Select human GST isoforms have also been shown to exert their protective effects through this ligandin-like behavior by binding with high affinity to inhibitory prostaglandins (e.g., PGJ_2), effectively sequestering them in the cytosol away from target nuclear proteins and preventing gene regulation (Paumi et al. 2004, Sanchez-Gomez et al. 2007). Lineweaver-Burke plots indicate that PGA_2 is a mixed inhibitor of *Cyphoma* GST activity, suggesting that this prostaglandin can bind to both the substrate active site and other sites on *Cyphoma* GSTs. This additional binding

capacity of PGA_2 to non-active site residues may indicate that sequestration of cyPGs by *Cyphoma* GSTs may be an important protective mechanism, and that over expression of GST forms could afford additional protection against prostaglandin-induced cytotoxicity.

The potential of GST to bind non-substrates has also been hypothesized to help protect consumers that regularly feed on allelochemical-rich prey. A comprehensive screening of allelochemicals from plants found that many inhibit the GSTs found in generalist lepidopterans (Yu & Abo-Elghar 2000), and over expression of GST enzymes in these consumers may play an important role in non-enzymatic cellular defense against dietary electrophilic chemical defenses. In marine systems, the chiton *Cryptochiton stelleri* regularly feeds on a red algal diet known to contain high concentrations of the feeding deterrent lanosol (DeBusk et al. 2000). Lanosol was found to be a noncompetitive inhibitor of *C. stelleri* GST activity, suggesting that while this feeding deterrent is not a substrate, overexpression of GSTs may serve as a detoxification strategy if GST enzymes can sequester the compound. Like *C. stelleri*, high constitutive GST activity was observed in *Cyphoma* independent of allelochemical diet and all of the gorgonian extracts examined contained potent inhibitory compounds. While the type of inhibition was not quantified for the gorgonian extracts, it is likely that gorgonian diets contain both substrates (i.e., PGA_2) and non-substrate ligands. Therefore, constitutive expression of GSTs may be indicative of a more *general* biochemical resistance strategy that is capable of responding to a diversity of compounds in the diet of a generalist consumer.

The results of this study provide the first comprehensive evaluation of the influence of dietary allelochemicals on the expression and function of glutathione transferases in a generalist marine consumer. Controlled feeding assays showed that *Cyphoma* digestive gland GST composition and activity did not vary with gorgonian diet. This result, in combination with evidence from *in vitro* inhibition studies with *Cyphoma* GSTs by gorgonian extracts, suggests the high constitutive expression of GST enzymes in *Cyphoma* digestive gland may be necessitated by the presence of numerous potent inhibitors/substrates in their gorgonian diets. Furthermore, all three prostaglandin series (A, E, F) found in the gorgonian *P. homomalla* were able to inhibit *Cyphoma* CDNB-conjugating GST activity, with relative potencies positively correlated with their abundance in gorgonian tissues. Kinetic analysis also demonstrated that inhibition of *Cyphoma* GSTs by prostaglandin A_2 was competitive toward CDNB and that its K_i (apparent K_m) values were similar to K_m values from known prostaglandin-conjugating vertebrate GSTs, suggesting that this allelochemical is a substrate for *Cyphoma* mu-class GSTs. Together, these findings suggest *C. gibbosum* detoxification enzymes have evolved to enable the detoxification of a broad range of electrophilic allelochemicals resulting from its close association with diverse gorgonian diets. Given the importance of allelochemicals in shaping patterns of predation and herbivory in marine systems, these findings suggest that co-evolved marine consumers have the capacity to detoxify allelochemicals in their prey, providing these consumers with a competitive advantage in ecosystems where allelochemically-rich prey species abound.

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Table 1. Effect of prostaglandin inhibitors on *Cyphoma gibbosum* affinity-purified GSTs

Inhibitor	IC_{50} value (μM)[a]	R^2	K_i (μM)[b]
15(S)-PGA_2	75.4	0.946	19.9
15(R)-15-methyl PGA_2	136.7	0.930	36.2
15(S)-PGE_2	312.2	0.821	82.6
15(S)-$PGF_{2\alpha}$	334.6	0.848	88.6

[a] The IC_{50} value is the concentration of inhibitor giving 50% inhibition of the enzyme activity assayed at pH 7.5 and 25°C, with 1 mM CDNB and 1 mM GSH and 4% (v/v) DMSO. The mean percent inhibition from snail affinity-purified GST concentrates (n = 5) was used to calculate IC_{50} values.
[b] K_i values were calculated using the Cheng-Prusoff equation with an average K_m value of 0.36 mM for CDNB (see Table 5); a CDNB substrate concentration of 1 mM; and IC_{50} values listed in the above table.

Table 2. Apparent Michaelis-Menten constants (K_m and V_{max}) for *Cyphoma gibbosum* affinity-purified GSTs with 1-chloro-2,4-dinitrobenzene

		1-Chloro-2,4-dinitrobenzene (CDNB)	
Experiment	15(S)-PGA_2 (μM)	K_m (mM)[a]	V_{max} (nmol/min/mL)[a]
1	--	0.38 ± 0.11	4310 ± 209
	60	3.41 ± 1.7	6025 ± 1354
2	--	0.22 ± 0.14	3401 ± 255
	200	22.7 ± 35	11631 ± 14685

[a] Apparent kinetic parameters reported

Reactions were carried out at 25°C in 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM GSH, 1 mM CDNB and 4% (v/v) DMSO. Values (means ± SEM) were obtained by regression analysis.

Table 3. Comparing models of 15(S)-PGA_2 inhibition of *C. gibbosum* affinity-purified GSTs by extra sum-of-squares (*F*-test)

		Model 2 - Michaelis-Menten (Alternative)			
	Model 1 (H_0)	df	*F*	p	Favored model
Experiment 1[a]	Competitive inhibition	1, 32	2.462	0.126	Null (competitive)
	Noncompetitive inhibition	1, 32	10.88	0.002	Alternative (Michaelis-Menten)
	Uncompetitive inhibition	1, 32	13.7	<0.001	Alternative (Michaelis-Menten)
Experiment 2[b]	Competitive inhibition	1, 31	2.471	0.126	Null (competitive)
	Noncompetitive inhibition	1, 31	14.05	<0.001	Alternative (Michaelis-Menten)
	Uncompetitive inhibition	1, 31	17.57	<0.001	Alternative (Michaelis-Menten)

[ab] Enzyme activity was assayed at pH 7.5 and 25°C with 1 mM GSH and varying concentrations of CDNB in the presence or absence of 15(S)-PGA_2 (60 μM^a or 200 μM^b).

Table 4. Comparing models of 15(S)-PGA_2 inhibition of *C. gibbosum* affinity-purified GSTs using Akaike information criterion

		Model 2 - Competitive inhibition[c]			
	Model 1 (Alternative)	ΔAIC_c	*P* (Model 1)[d]	*P* (Model 2)[d]	Favored model
Experiment 1[a]	Noncompetitive inhibition	-7.864	1.92%	98.08%	Model 2 (competitive)
	Uncompetitive inhibition	-10.16	0.62%	99.38%	Model 2 (competitive)
Experiment 2[b]	Noncompetitive inhibition	-10.4	0.55%	99.45%	Model 2 (competitive)
	Uncompetitive inhibition	-13.03	0.15%	99.85%	Model 2 (competitive)

[ab] Enzyme activity was assayed at pH 7.5 and 25°C with 1 mM GSH and varying concentrations of CDNB in the presence or absence of 15(S)-PGA_2 (60 μM[a] or 200 μM[b]).

[c] Model 2 (competitive inhibition) is considered the most simple form of inhibition based on the number of parameters in the model in comparison to model 1.

[d] *P* = probability of the model given the data

Table 5. Kinetic constants for *C. gibbosum* affinity-purified GSTs with 15(S)-PGA_2 obtained by fitting to the competitive inhibition equation

Inhibitor	Inhibitor (μM)	1-Chloro-2,4-dinitrobenzene (CDNB)		
		K_m (mM)[a]	V_{max} (nmol/min/mL)	K_i (μM)
15(S)-PGA_2	60	0.47 ± 0.17	4494 ± 286	24.2 ± 10
	200	0.25 ± 0.15	3462 ± 263	15.6 ± 9.1

Table reports the results of two separate experiments using either 60 μM or 200 μM of the inhibitor (15(S)-PGA_2) with varying concentrations of CDNB.

Reactions were carried out at 25°C in 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM GSH and 4% (v/v) DMSO. Values (means ± SEM) were obtained by regression analysis.

[a] An average K_m value of 0.36 mM for CDNB was used to calculate K_i values in Table 1.

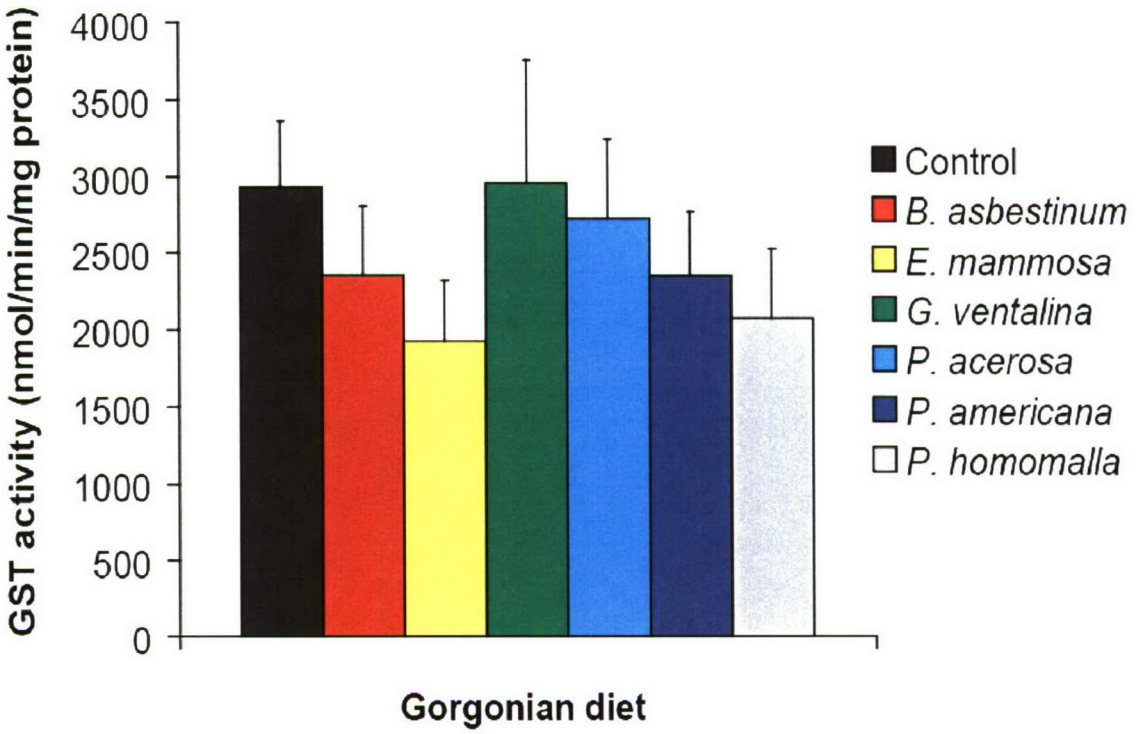


Figure 1. GST activity levels from digestive gland cytosolic preparations of *C. gibbosum* feeding either on a control diet or six gorgonian diets in controlled 4 d feeding assays. Bars represent the mean GST activity (± SE) of snails feeding on control diet (n = 12 snails; e.g., alginic acid + squid powder) or one of six gorgonian diets (*B. asbestinum*, n = 4; *E. mammosa*, n = 4; *G. ventalina,* n = 4; *P. acerosa*, n = 4; *P. americana*, n = 7; *P. homomalla*, n = 4). The reaction mixture contained 2 µg of cytosolic protein in 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM GSH and 1 mM CDNB at 25°C. Results indicate that GST activity did not significantly differ among snail diets (ANOVA, p = 0.687) or between snails collected from different reefs feeding on the control diet (ANOVA, p = 0.109).

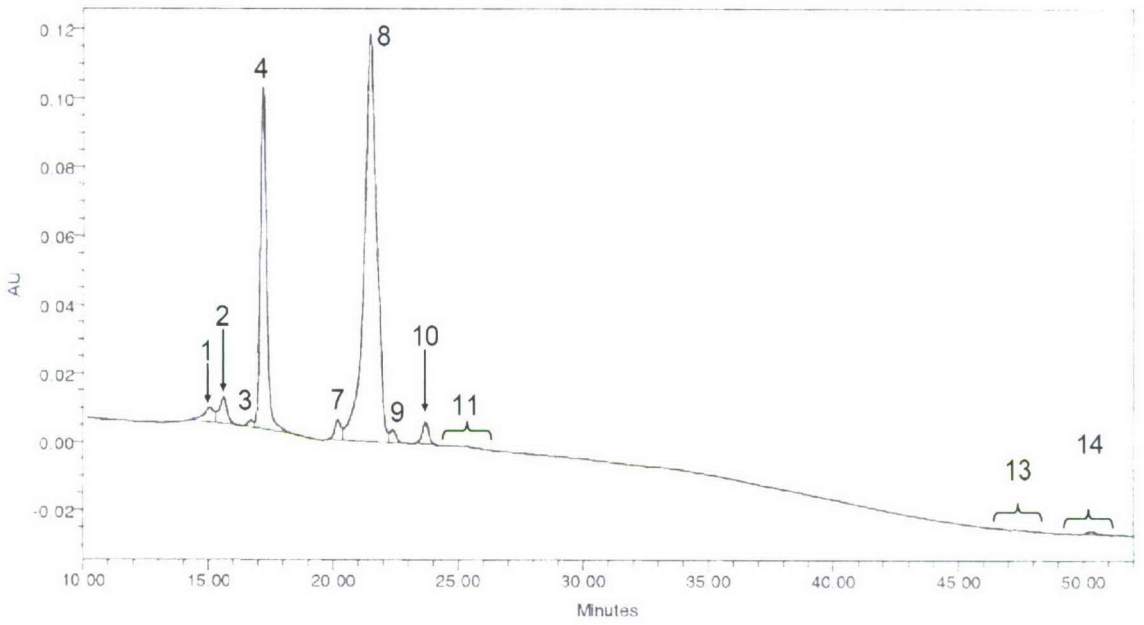


Figure 2. Representative HPLC separation of affinity-purified GST subunits from a *C. gibbosum* digestive gland. Representative HPLC separation of an affinity-purified extract from an individual *C. gibbosum* feeding on *B. asbestinum*. GST subunits were separated on a reverse phase VYDAC protein/peptide column (C18 μm 250 mm x 2.1 mm) with a flow rate of 0.5 mL min^{-1}. Mobile phase A consisted of acetonitrile/water/TFA (38:62:0.1, v/v) and mobile phase B consisted of acetonitrile/water/TFA (80:20:0.1, v/v). GST subunits were separated using a linear gradient from 0 to 40% B in 22 min, and 40 to 100% B in 37 min and visualized at 214 nm. Fourteen unique peaks were identified; however, not all were visible in one HPLC spectrum, therefore a representative spectrum was chosen. The position of HPLC peaks 1-4, 7-11, 13 and 14 are labeled.

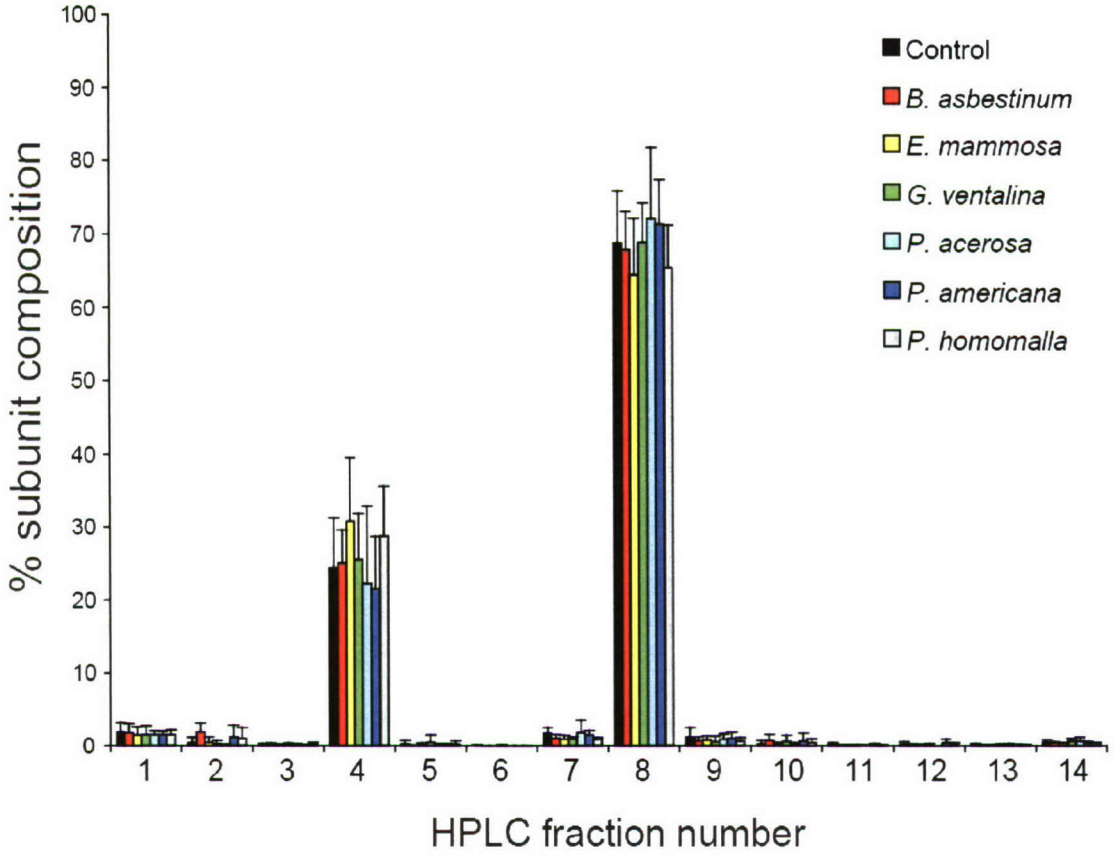


Figure 3. Average GST subunit percent composition as a function of snail diet. Bars represent the mean percent GST subunit composition (± SD) in affinity-purified digestive gland preparations from snails grouped by diet; control diet (n = 12 snails) and gorgonian diets (*B. asbestinum*, n = 4; *E. mammosa*, n = 4; *G. ventalina,* n = 4; *P. acerosa*, n = 4; *P. americana*, n = 7; *P. homomalla*, n = 4). Statistical analysis (ANOVA) indicated that GST subunit percent composition did not significantly differ among snail diets.

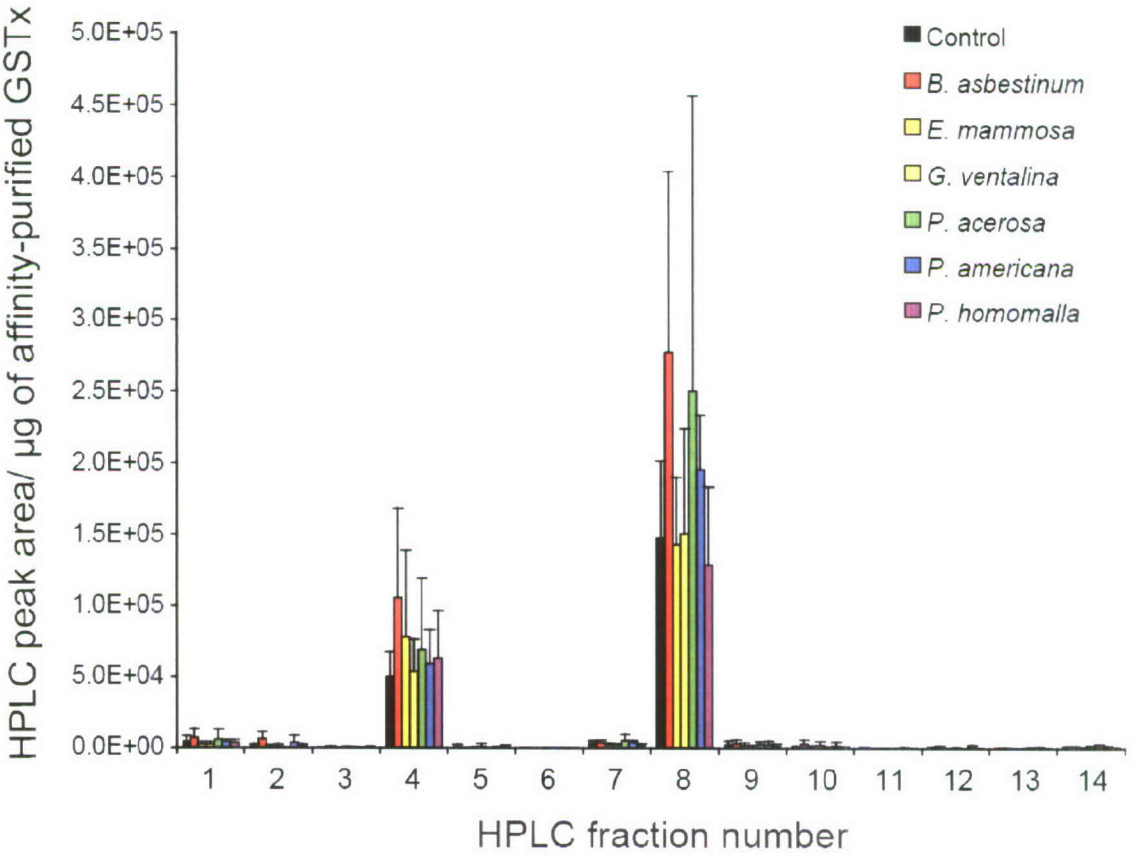


Figure 4. Peak area normalized to the amount of affinity-purified GST sample applied to the HPLC column. Bars represent the mean HPLC peak area normalized for the amount of GST protein applied to the HPLC column as a function of snail diet (± SE); control diet (n = 12 snails) and gorgonian diets (*B. asbestinum*, n = 4; *E. mammosa*, n = 4; *G. ventalina*, n = 4; *P. acerosa*, n = 4; *P. americana*, n = 7; *P. homomalla*, n = 4). Statistical analysis (ANOVA) indicated the amount of starting material did not significantly affect GST subunit abundance among snail diets.

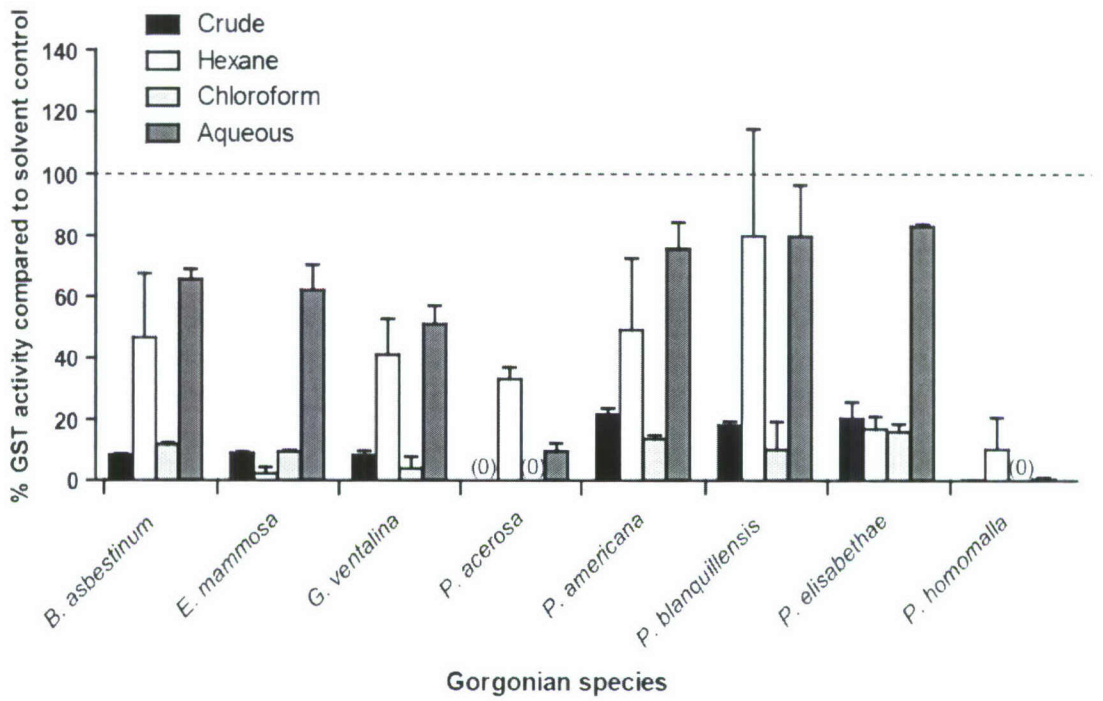
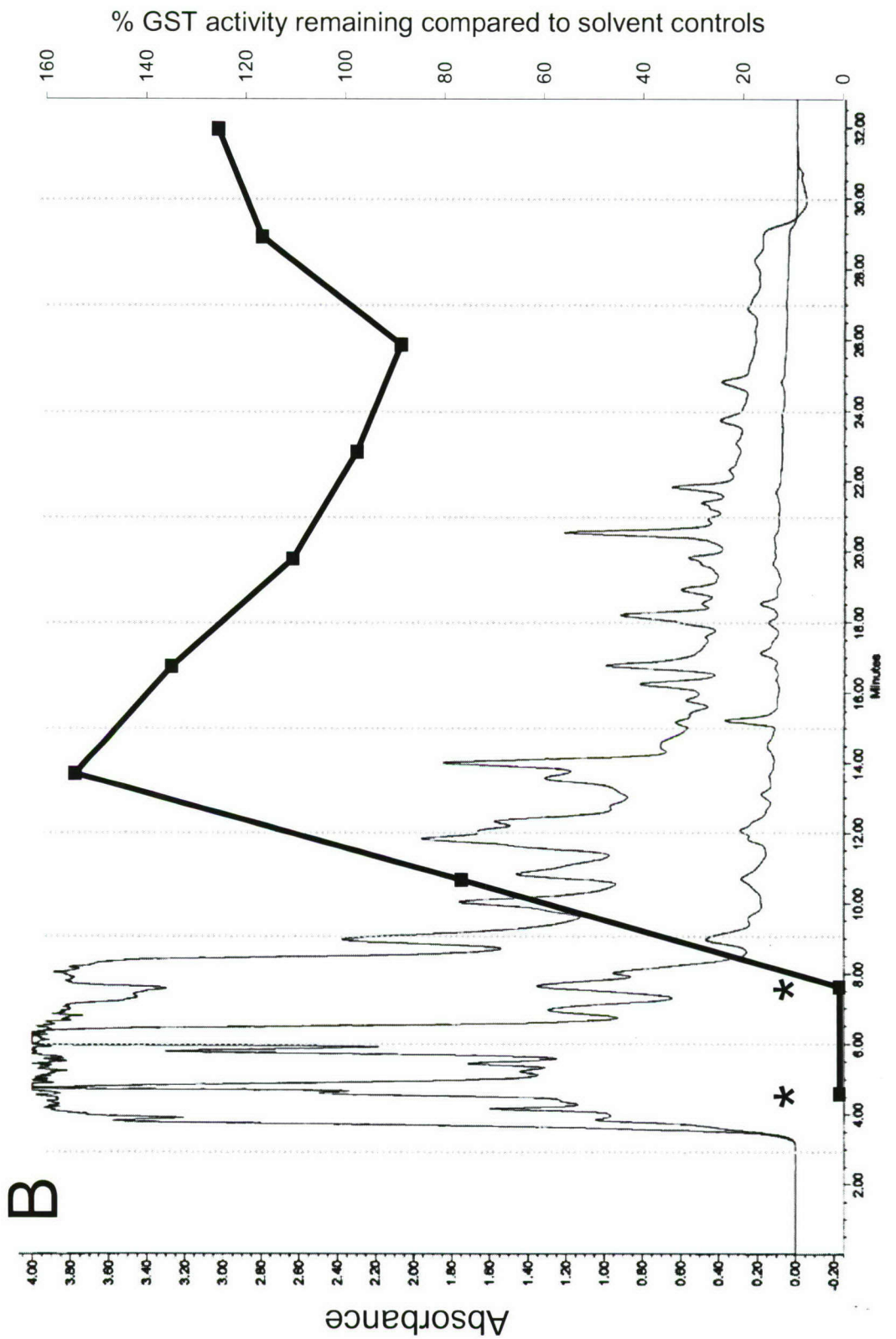
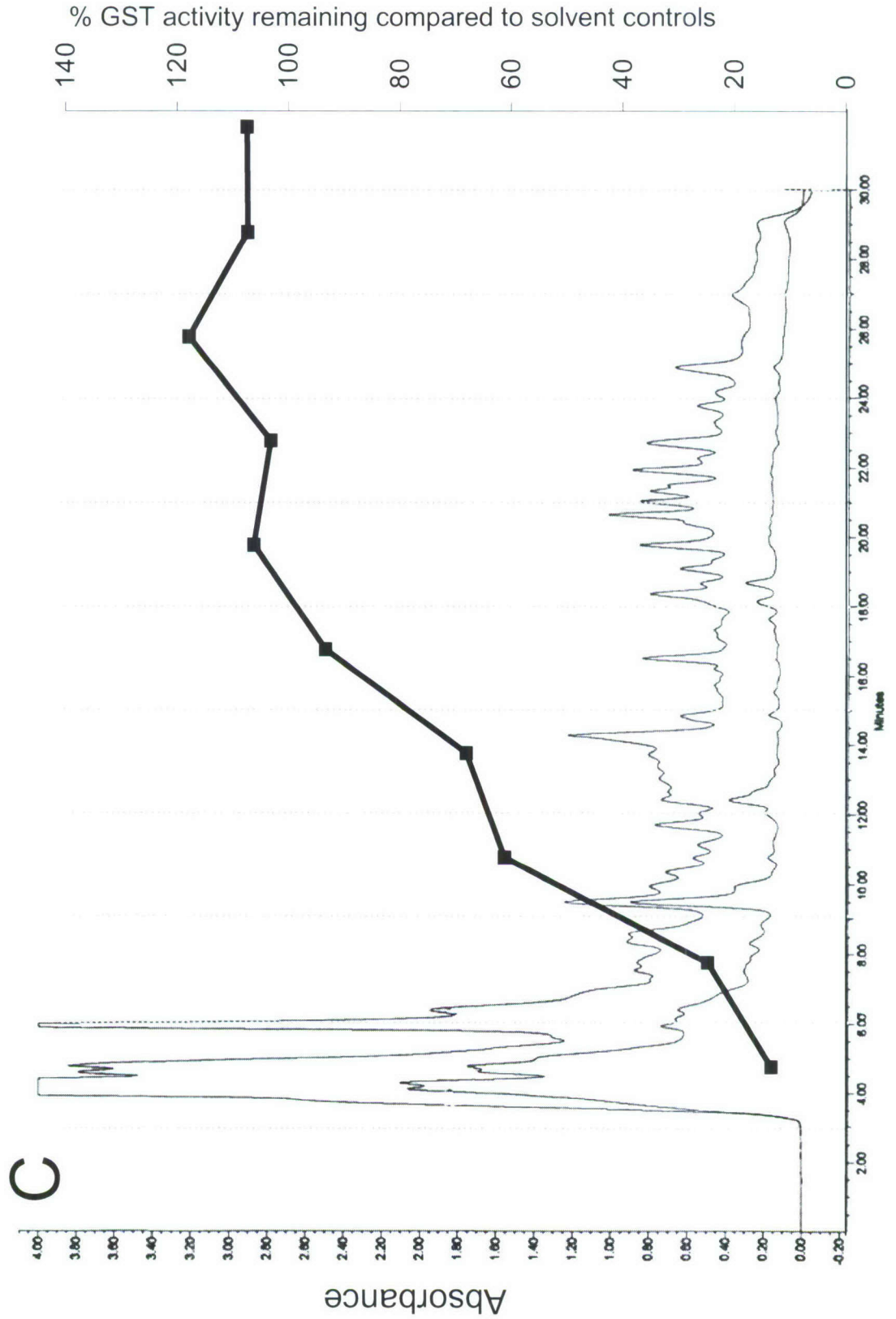


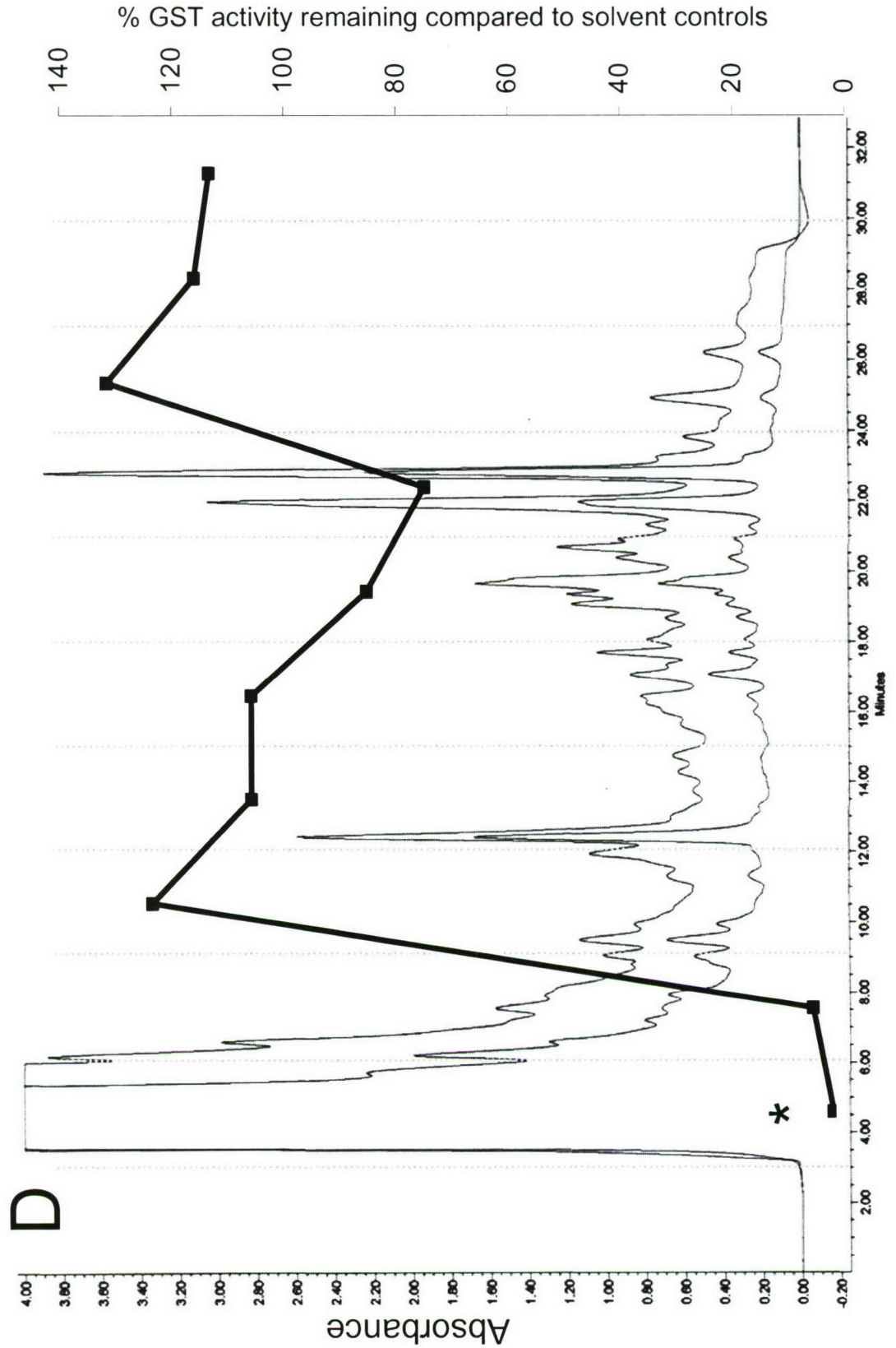
Figure 5. Inhibition of *C. gibbosum* GST activity by gorgonian crude and semi-purified extracts. Bars represent the mean (± SE) percent GST activity remaining after exposure to gorgonian compounds compared to solvent controls. Reaction mixture consisted of 2 µg cytosolic protein in 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM GSH, 1 mM CDNB at 25°C with <0.01% (v/v) solvent. Reactions were performed in duplicate with digestive gland crude cytosolic preparations from two snails. Crude extracts from eight gorgonian species were tested at 1/20th natural volumetric concentration (NC) found in gorgonian tissues for their ability to inhibit crude cytosolic GST activity. Hexane and aqueous soluble extracts were tested at 1/4 x NC. Chloroform soluble fractions were tested at 1/4 x NC with the following exceptions: *G. ventalina*, *P. acerosa*, and *P. homomalla* were tested at 1/8 x NC. A zero indicates complete inhibition of GST activity by the gorgonian extract or fraction. Bars approaching the dotted line indicate no difference from solvent controls.

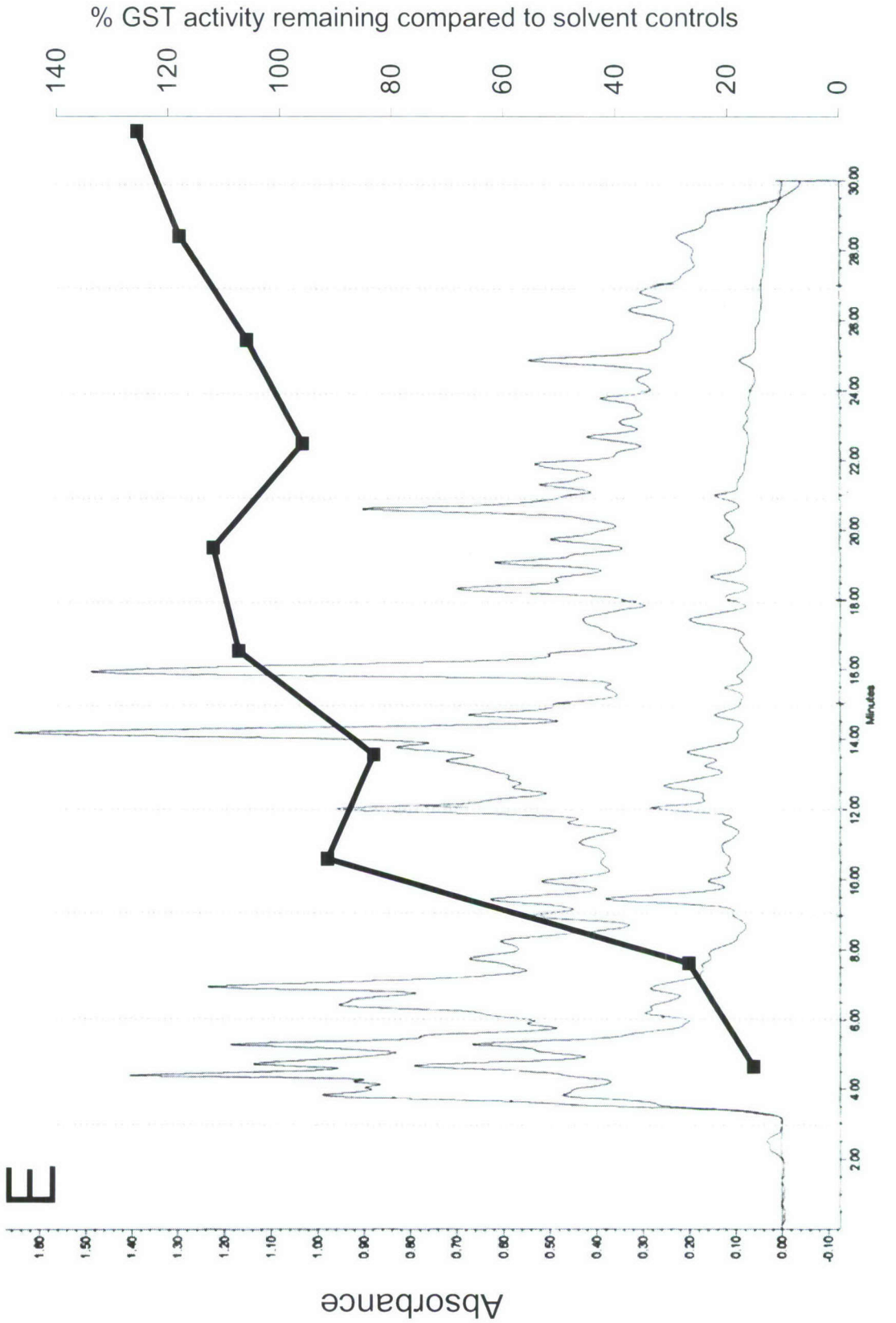
Figure 6. Inhibition of *C. gibbosum* GST activity by the chloroform-soluble HPLC fractions from eight gorgonian species. Chloroform partitions from eight gorgonian species ((**A**) *B. asbestinum*; (**B**) *E. mammosa*; (**C**) *G. ventalina*; (**D**) *P. acerosa*; (**E**) *P. americana*; (**F**) *P. blanquillensis;* (**G**) *P. elisabethae*; (**H**) *P. homomalla*) were separated into ten fractions (indicated by dotted lines) using a reverse-phase HPLC column (Zorbax SB-C18, 9.4mm x 2cm; solvent flow rate = 3 mL/min; injection volume = 500 μL). Mobile phase: methanol/water 9:1 from 0-5 mins; linear ramping to 100% methanol from 5-18 min; 100% methanol from 18-25 min; linear gradient to initial starting conditions of methanol/water 9:1 from 25-26 min; column flushed with methanol/water 9:1 from 26-33 min. Absorbance was monitored at 215 and 254 nm and fractions were collected every three minutes beginning at t_o=3min. Overlaid on the HPLC absorbance spectra are the results GST inhibition assays with affinity-purified GST protein. The reaction mixture consisted of 6 ng of affinity-purified GST protein in 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM GSH, 1 mM CDNB at 25°C with 2% (v/v) methanol. An affinity-purified GST preparation from a single digestive gland was used as the protein source with a specific activity (mean ± SE) of 561 ± 25 μmol min^{-1} mg $protein^{-1}$. HPLC fractions were tested at $1/10^{th}$ natural volumetric concentration, unless marked by an asterisk indicating samples were further tested at $1/200^{th}$ natural volumetric concentration. Each point (■) represents the mean of two technical replicates.

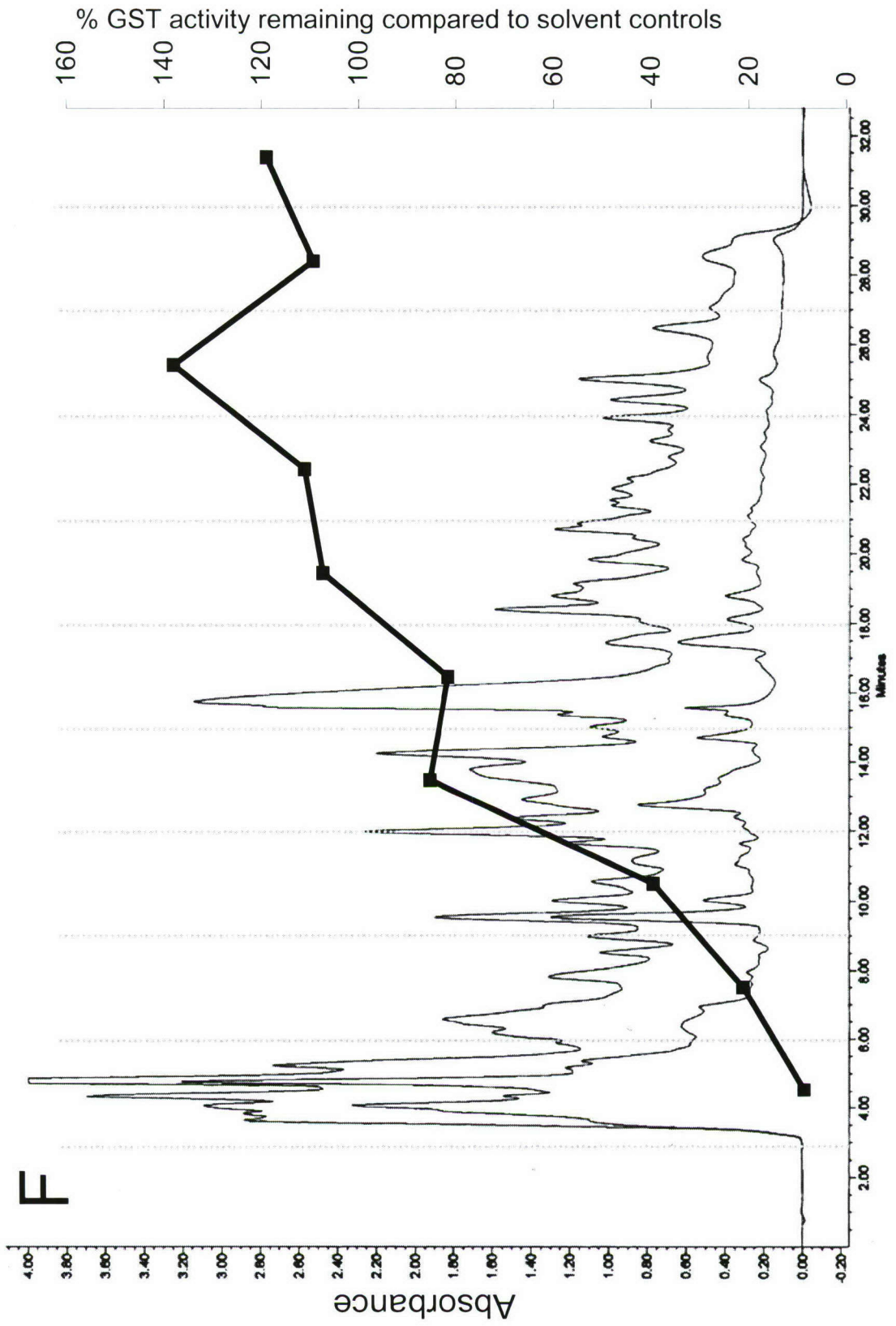
% GST activity remaining compared to solvent controls
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HPLC Fractions


B
% GST activity remaining compared to solvent controls
Absorbance
Minutes
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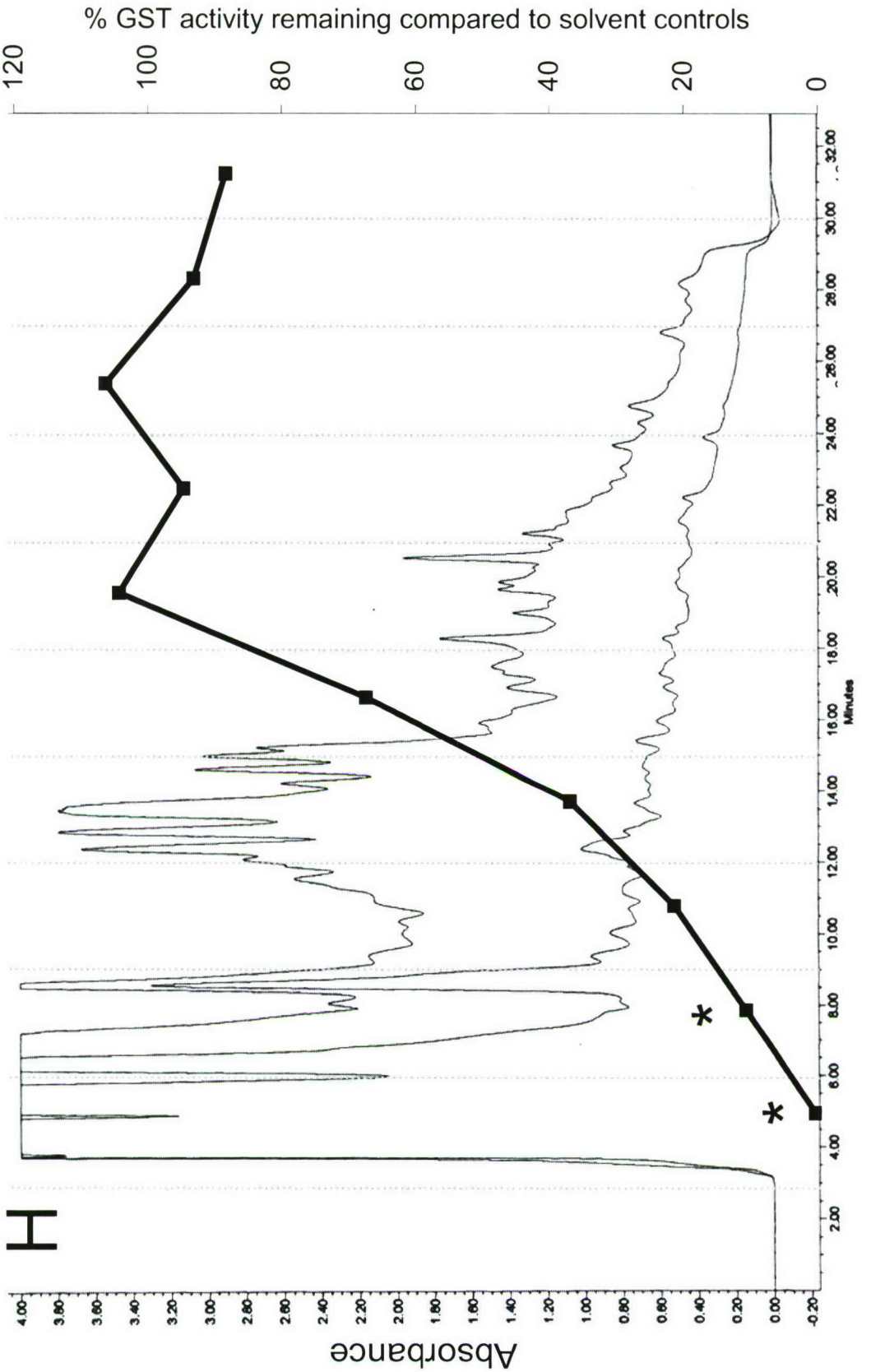

% GST activity remaining compared to solvent controls
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Absorbance
Minutes


% GST activity remaining compared to solvent controls
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% GST activity remaining compared to solvent controls
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% GST activity remaining compared to solvent controls
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% GST activity remaining compared to solvent controls
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% GST activity remaining compared to solvent controls
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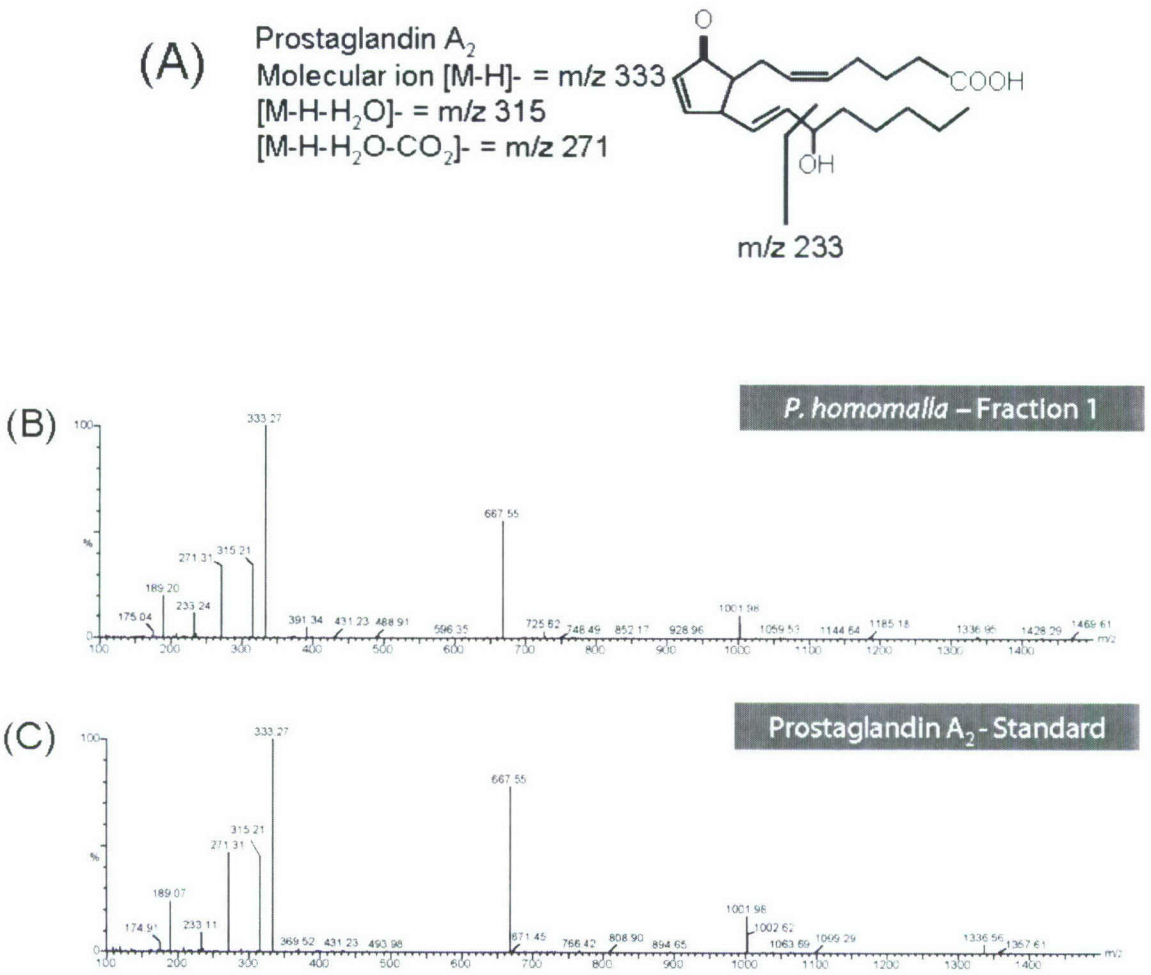


Figure 7. LC-MS identification of PGA_2 in *P. homomalla* chloroform extracts. The fragmentation pattern of the free hydroxy acid of PGA_2 is shown in (**A**). The similarity between the full-scan mass spectrum of *P. homomalla* fraction 1 (**B**) and the PGA_2 standard (mol wt 333) (**C**) indicates that the *P. homomalla* chloroform-soluble HPLC fraction 1 contains PGA_2.

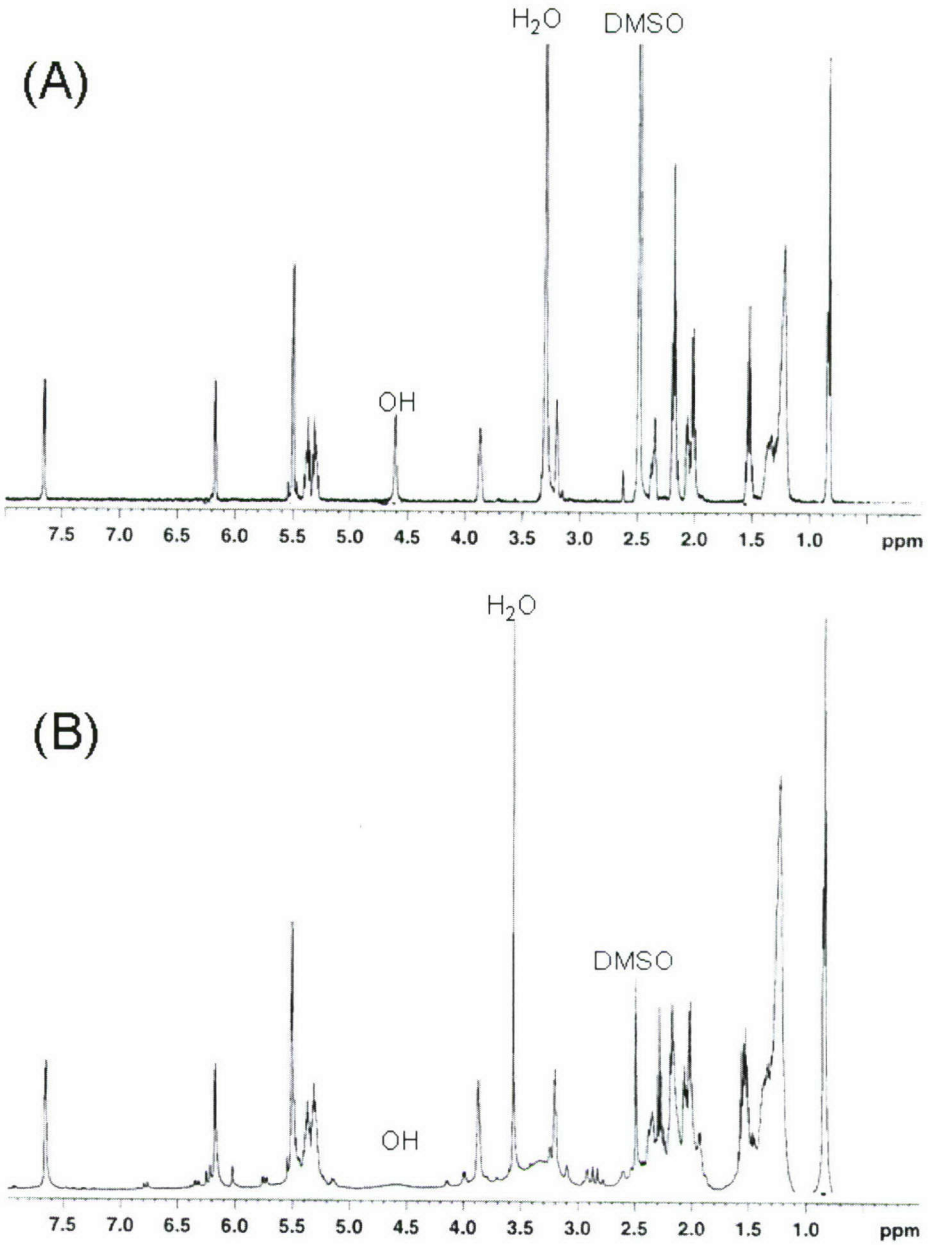


Figure 8. Comparison of ^{1}H NMR spectra from the PGA_2 standard (A) and the chloroform-soluble fraction 1 from *P. homomalla* (B).

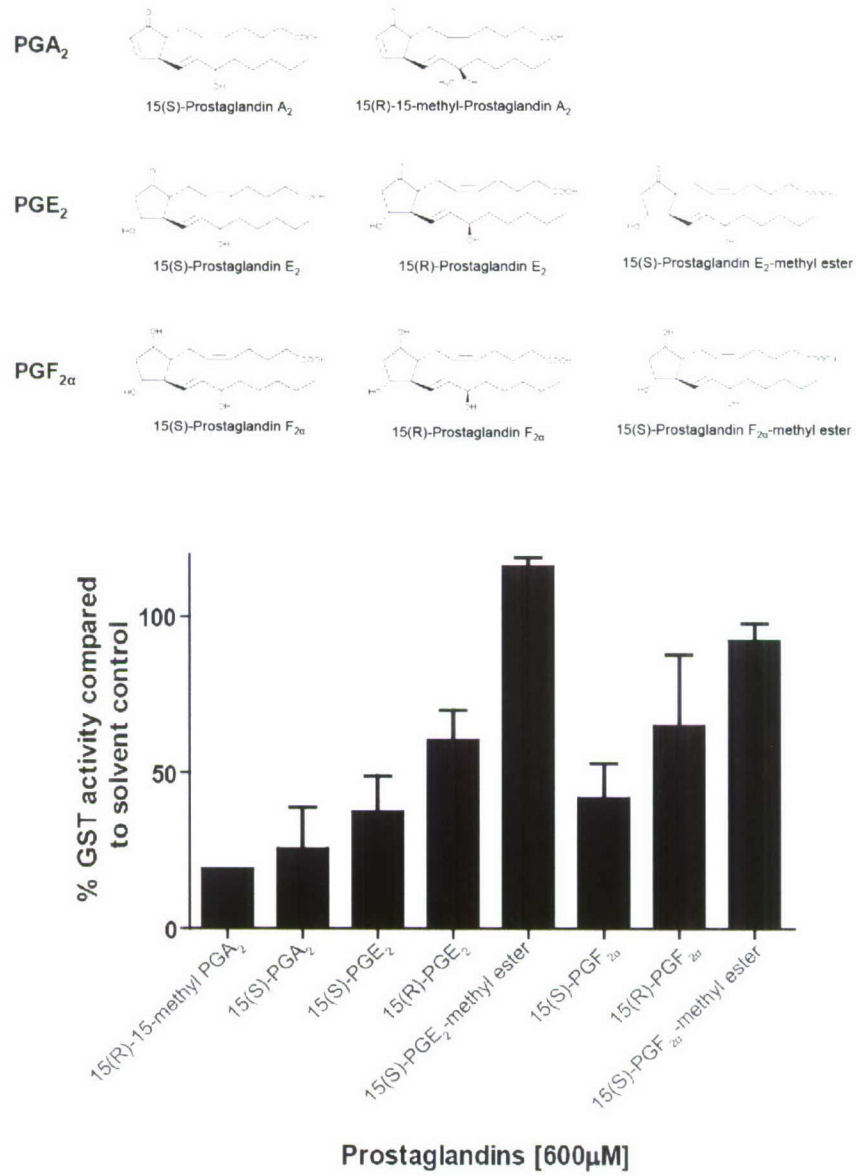


Figure 9. Effect of prostaglandin A, E, and F series on cytosolic *C. gibbosum* GST activity. Eight commercially available prostaglandin compounds, representing a diversity of prostaglandin series present in gorgonian tissues, were screened for their ability to inhibit GST activity at 600 μM. Bars represent the mean (± SE) percent GST activity remaining after prostaglandin exposure compared to solvent controls. The reaction mixture consisted of 2 μg of cytosolic GST protein in 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM GSH, 1 mM CDNB at 25°C with 5% (v/v) DMSO. Digestive gland cytosolic preparations from two snails were used as the enzyme source with specific activities of 5.8 and 5.1 μmol min^{-1} mg $protein^{-1}$, respectively.

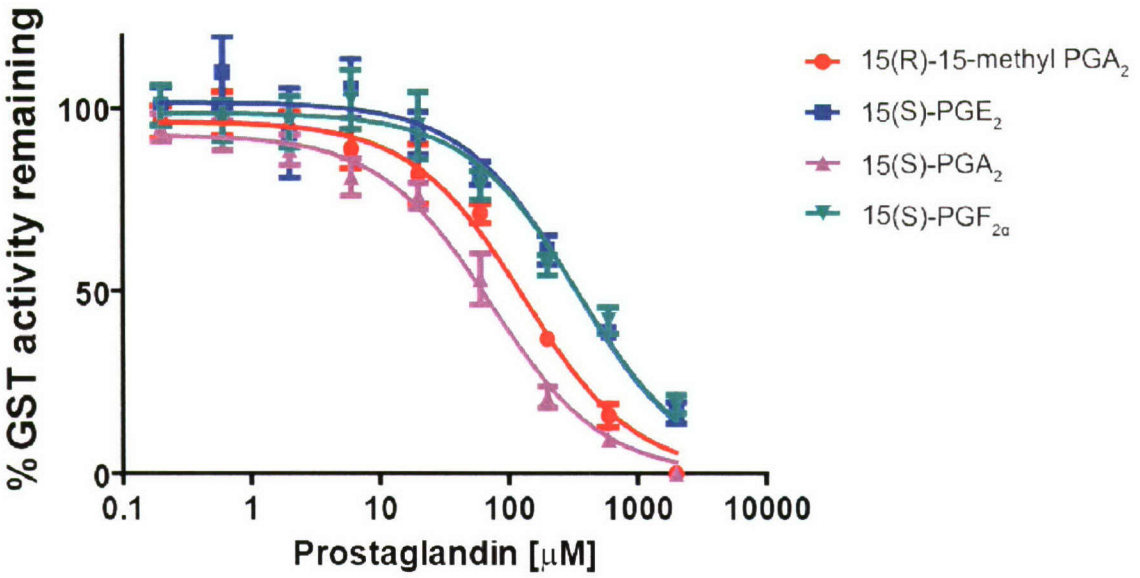


Figure 10. Inhibition of *C. gibbosum* affinity-purified GST activity by selected prostaglandins. Points represent the mean (± SE) GST activity remaining compared to solvent controls after incubation with various concentrations (0.2 – 2000 μM) of 15(R)-15-methyl PGA_2 (●), 15(S)-PGE_2 (■), 15(S)-PGA_2 (▲), 15(S)-$PGF_{2\alpha}$ (▼). The reaction mixture consisted of 3.3 – 6.4 ng of affinity-purified GST protein in 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM GSH, 1 mM CDNB at 25°C with 5% (v/v) DMSO. Affinity-purified GST preparations from five snails were used as the enzyme source with specific activities of ranging from 290 – 582 μmol min^{-1} mg $protein^{-1}$. Plots were used to obtain the IC_{50} value for each prostaglandin using Prism software and are listed in Table 1.

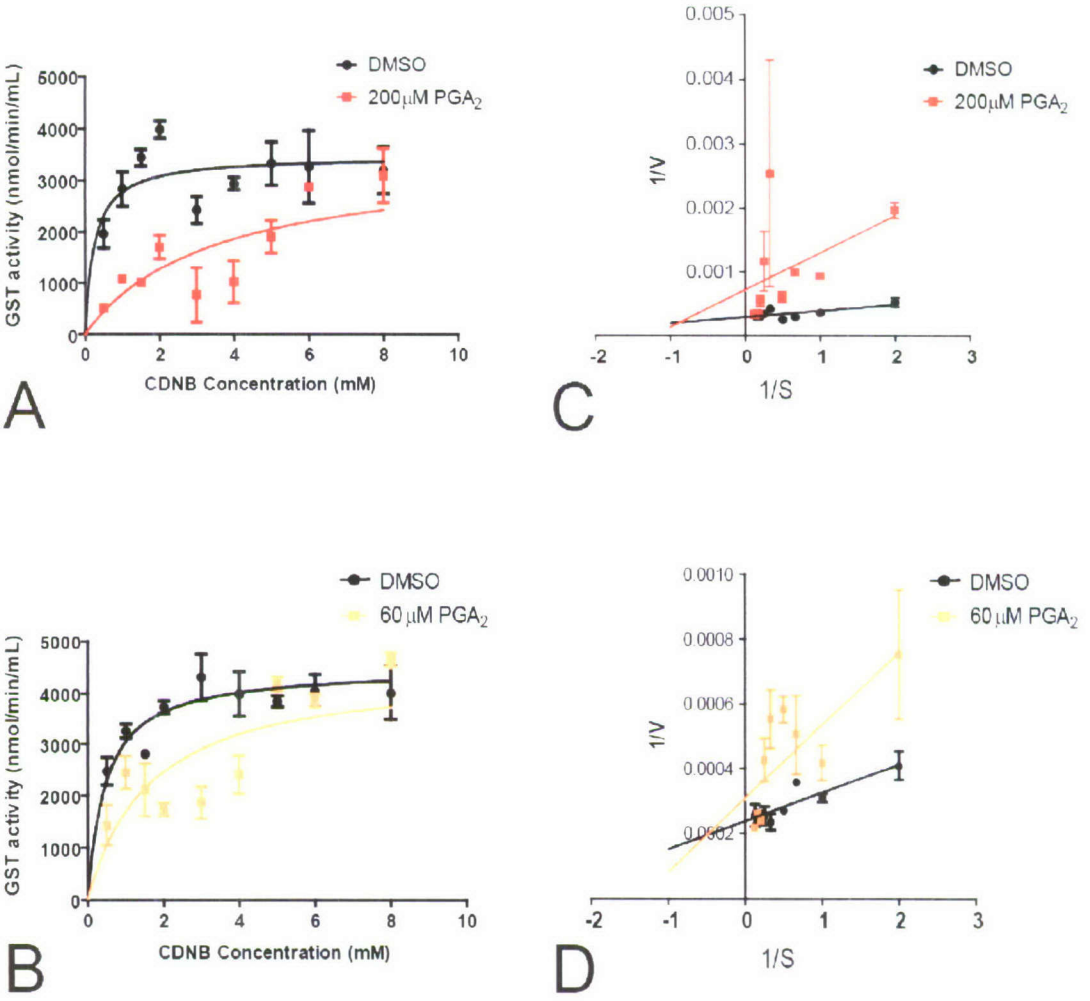


Figure 11. Lineweaver-Burke plots showing mixed inhibition of *Cyphoma* GSTs toward CDNB by 15(S)-PGA_2. GST activity was measured at varying concentrations of CDNB in the absence (DMSO) and presence of 200 μM (**A**) and 60 μM (**B**) PGA_2. The reaction mixture consisted of varying concentrations of CDNB and 6 ng of affinity-purified GST protein in 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM GSH at 25°C with 5% (v/v) DMSO. An affinity-purified GST preparation from a single digestive gland was used as the protein source with a specific activity of 561 ± 25 μmol min^{-1} mg $protein^{-1}$. DMSO (●) and PGA_2 (■) values at each CDNB concentration represent the mean (± SE) of two technical replicates. Lineweaver-Burke plots (**C, D**) of $1/V_{max}$ versus CDNB concentration were constructed from data in part A and B, respectively. Lineweaver-Burke plots indicated a mixed type of inhibition (i.e., competitive and noncompetitive inhibition).

Chapter V

The role of multixenobiotic transporters in generalist and specialist molluscan predators as counter-defense mechanisms against dietary chemical stress

Abstract

Multixenobiotic transporters have been extensively studied for their ability to modulate the absorption, metabolism, and toxicity of pharmacological agents. These transporters may also play critical roles in regulating the efflux or accumulation of natural dietary toxins in marine consumers, perhaps ultimately influencing dietary choice. This study presents functional and molecular evidence for multixenobiotic transporter-mediated efflux activity and expression in a generalist (*Cyphoma gibbosum*) and specialist (*Tritonia hamnerorum*) molluscan predator, both of which are obligate consumers of chemically defended gorgonian corals. Immunochemical analysis revealed that proteins with homology to the permeability glycoprotein (Pgp) subfamily of ABC transporters were not expressed in *C. gibbosum* digestive gland preparations or tissues, but were highly expressed in *T. hamnerorum* tissue homogenates. Pgp expression in *T. hamnerorum* was primarily localized to the apical tips of the gut epithelium, a region particularly vulnerable to dietary allelochemical exposure, suggesting these transporters may provide an effective means of protection against ingested environmental toxins. *In vivo* dye assays with *T. hamnerorum* indicated concentration-dependent inhibition of calcein-am efflux by specific inhibitors of Pgp and the multidrug resistance-associated protein (MRP), implying that both ABC protein subfamilies may facilitate allelochemical transport in this specialist predator. An adaptor-ligated cDNA library with degenerate and specific primers was used to clone eight cDNAs encoding two partial ABCB and two partial ABCC proteins from both *C. gibbosum* and *T. hamnerorum*. In addition, real-time quantitative PCR analysis demonstrated selected *C. gibbosum* MRP transcripts were

constitutively expressed in the digestive gland regardless of allelochemical diet. The constitutive expression of *C. gibbosum* MRP transporters with homology to mammalian glutathione-conjugate transporters may be explained by previous evidence suggesting that gorgonian diets contain high affinity substrates for phase II detoxification enzymes, whose conjugates may in turn be exported by MRPs. In summary, the foraging strategies of marine consumers may result in different expression patterns of related multixenobiotic transporters depending on diet chemistry and the requirements of the consumer; however, it is likely that both subfamilies of ABC transporters operate as effective allelochemical pumps, providing a physiological defense against environmental toxicants in both molluscan species.

Introduction

Soft-bodied benthic organisms produce a vast number of structurally diverse secondary metabolites, many of which function as feeding deterrents in marine systems (Hay & Fenical 1988, Harvell & Fenical 1989, Paul 1992, Hay 1996, Stachowicz 2001). For marine consumers, the consequences of ingesting chemically defended prey can be quite severe (Targett & Arnold 2001), yet specialized consumers that solely feed on toxic prey can apparently tolerate these dietary compounds, and in some cases, even concentrate the defensive compounds for their own protection (Cimino et al. 1985, Cronin 2001). Few studies have explored the physiological targets of these compounds in generalist consumers and progress has been slow in understanding the mechanisms by which more specialized predators are able to cope with their toxic prey.

For organisms that feed on toxin laden prey, multixenobiotic resistance transporters represent one mechanism by which predators may protect themselves against the toxicity of their prey, either by actively exporting allelochemicals out of cells or sequestering toxins within specialized cells or organelles, effectively compartmentalizing them away from vulnerable protein and DNA targets (Sorensen & Dearing 2006). Those proteins involved in the transport of xenobiotics across membranes belong to the ATP Binding Cassette (ABC) family, and likely provide consumers with a 'first line of defense' against the accumulation of dietary allelochemicals (Epel 1998). Several members of the ABCB (P-glycoprotein; Pgp or MDR) and ABCC (multidrug resistance-associated protein or MRP) subfamilies function as highly promiscuous transporters, capable of trafficking a diverse array of moderately hydrophobic xenobiotics across cell membranes (Bodo et al. 2003). Together, the overexpression of both Pgp and MRP in tumor cell lines has long been known to mediate the ATP-dependent efflux of anticancer agents, conferring tumor resistance to natural product chemotherapeutic compounds (Deeley & Cole 2006, Sarkadi et al. 2006). Only recently has it been suggested that ABC transporters are responsible for regulating the absorption of allelochemicals in the guts of consumers, and may therefore have a significant influence on the foraging patterns and ultimately diet choice of these organisms (Sorensen & Dearing 2006, Sotka & Whalen 2008).

The ubiquity of these multixenobiotic resistance proteins (MXRs) in aquatic organisms has been confirmed by immunological cross-reactivity studies, *in vivo* functional assays, competitive inhibition substrate binding assays (Bard 2000, Eufemia &

Epel 2000), and more recently by molecular evidence (Goldstone et al. 2007). The distribution of MXRs in tissues involved in absorption, secretion and barrier functions in aquatic invertebrates (Bard 2000) suggests that they may play a role in the prevention of dietary xenobiotic absorption. Several studies have also identified marine natural products from algae, tunicates, sea hares and gorgonians that may be substrates for multixenobiotic resistance proteins (Suganuma et al. 1988, Chambers et al. 1993, Williams & Jacobs 1993, Aherne et al. 1996, Quesada et al. 1996, Schroder et al. 1998, Tanaka et al. 2002), suggesting that the pool of potential substrates in marine ecosystems may be extensive. Given this evidence, the constitutive or inducible expression of MXRs in marine consumers may serve a protective role against a range of allelochemically-rich prey.

Analogous to the reciprocal evolution of terrestrial consumer biotransformation enzymes in response to their toxic host plants (Li et al. 2004), marine consumers likely evolved multixenobiotic resistance mechanisms out of a similar need to mount a cellular defense against the escalating chemical attack produced by their prey/host(s). As a result of this chemical 'arms race', it is expected that many marine allelochemicals may be MXR substrates or potent inhibitors (Smital et al. 2004) – the latter likely functioning as counter-defense strategies of chemical defended prey to thwart the detoxification attempts by their consumers (Sorensen & Dearing 2006). Eufemia et al. (2002) examined seaweed and phytoplankton extracts co-occurring with the filter feeder, *Mytilus californianus*, for their ability to act as chemosensitizers (substrates and/or inhibitors) for molluscan ABC transporters using competition-based fluorescent dye assays. Tissue

accumulation of the fluorescent ABC transporter substrate (e.g., rhodamine) occurred if the test compound was a substrate (or inhibitor) of transport activity. Moderately hydrophobic fractions from five algal extracts caused the greatest accumulation of rhodamine dye in the gill tissue of *M. californianus*, suggesting the presence of P-glycoprotein substrates in algal species. In addition, polyphenolics (phlorotannins), known to be potent feeding deterrents in some marine ecosystems (Targett & Arnold 2001), were also found to cause dye accumulation, suggesting they too are substrates or inhibitors for MXRs. Recent evidence also reveals that the unpalatability of newly invasive seaweeds may be linked in part to the liberation of potent MXR inhibitors (Smital et al. 2004). Extracts from the invasive green alga, *Caulerpa taxifolia*, resulted in the reversal of MXT activity in cell culture and in *in vivo* experiments with bivalves *Dreissena polymorpha* and *Mytilus galloprovincialis* (Smital et al. 1996, Schroder et al. 1998, Smital et al. 2004). Consumption of these algal species could pose severe threats to naïve consumers who may lack adequate adaptations to cope with the invasives. However, for those marine consumers that are regularly exposed to toxic allelochemicals in their diet, maintaining high levels of ABC transporter protein and/or activity in vulnerable tissues may allow these consumers to exploit an otherwise unattainable resource.

The objective of this study was to characterize the multixenobiotic resistance proteins potentially involved in dietary allelochemical resistance in two species of tropical gastropods that feed exclusively on allelochemically defended gorgonian corals. A combination of molecular, immunological and functional approaches were used to

examine the expression and activity of molluscan ABC transporters in *Tritonia hamnerorum*, a specialist nudibranch that feeds on a single genus of gorgonian, and *Cyphoma gibbosum*, a generalist gastropod that includes multiple gorgonian families in its diet. Evidence from terrestrial chemical ecology studies suggests that generalists have likely evolved a greater diversity of catalytically versatile xenobiotic resistance mechanisms as a result of their chemically diverse diets, compared to specialists that feed on a reduced spectrum of allelochemicals due to their narrow foraging range (Li et al. 2004, Sorensen & Dearing 2006). This study presents the initial characterization of those molluscan MXRs likely responsible for xenobiotic resistance, and will ultimately contribute to a more complete understanding of how generalists and specialists cope with their allelochemical diet(s).

Expression levels of select MXR transporters were compared between the two molluscan species to examine the relationship between MXR expression and diet specificity. Reverse transcription-polymerase chain reaction (RT-PCR) in combination with adaptor-specific and degenerate primers was used to perform targeted searches for ABC transporter genes and successfully identified two Pgp homologs and two MRP homologs from each molluscan species. To determine if MXR expression was influenced by the different allelochemical profiles of host gorgonians, quantitative RT-PCR was used to measure the expression of selected ABC transporter transcripts in digestive gland samples from *C. gibbosum* feeding on seven different gorgonian species. In addition, immunochemical analysis was used to characterize the expression and localization of Pgp in molluscan tissue homogenates and sections. Finally, quantification

of MXR activity was measured using *in vivo* competitive-based fluorescent dye assays by examining the sensitivity of *Tritonia* transporters to model Pgp and MRP inhibitors. Together, these results indicate that both ABC protein subfamilies may play a significant role in defending molluscan predators against naturally occurring dietary toxins.

Materials and Methods

Animal collection

In January 2004, over 200 *Tritonia hamnerorum*, ranging in size from 3 mm to 15 mm, were collected from shallow reefs surrounding the Perry Institute of Marine Science (PIMS), Lee Stocking Island, Exuma Cays, Bahamas. The purple sea fan, *Gorgonia ventalina*, was the only species of octocoral observed to serve as host for *Tritonia hamnerorum* at our study site. The density of *Tritonia hamnerorum* on individual sea fans ranged from two to over 500 individuals per sea fan. Nudibranchs were collected by removing the portion of sea fan housing them with scissors and transporting both the gorgonian and nudibranchs back to wet laboratory facilities provided by PIMS where they were maintained in flowing filtered seawater until further use. *T. hamnerorum* individuals used for RNA and protein isolation were removed from their host gorgonian, pooled, flash frozen and stored in liquid nitrogen until processing.

In a preliminary series of feeding assays conducted in January 2004, adult *Cyphoma gibbosum* were allowed to feed for four days on one of four gorgonian species (*Briareum asbestinum, Gorgonia ventalina, Pseudopterogorgia acerosa, Pseudopterogorgia americana*). A complete description of the feeding assay

experimental set-up can be found in Chapter 3, Methods. Upon completion of the assays, digestive glands were immediately dissected and either frozen in liquid nitrogen (n = 2 digestive glands) or stored in RNALater® at -80°C (n = 15 digestive glands) until further processing.

RNA isolation and RT-PCR cloning

Both *Tritonia hamnerorum* and *Cyphoma gibbosum* collected in 2004 provided tissue for initial ABC transporter cloning studies. Total RNA from a frozen, pooled sample of whole *T. hamnerorum* (267.9 mg; n > 40 individuals) was isolated with the RNeasy Maxi Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. RNA isolation from pooled *C. gibbosum* digestive glands was performed as described in Chapter 3, Methods. The integrity of the RNA samples was checked by electrophoresis on a 1% agarose-formaldehyde gel in MOPS buffer and visualized with ethidium bromide under UV light. Total RNA concentration was determined using a Nanodrop spectrophotometer (Wilmington, DE). Poly(A)+ RNA was purified using the MicroPoly(A)Purist mRNA purification kit (Ambion, Austin, TX) according to the manufacturer's instructions. First-strand cDNA was reverse transcribed from 2 μg poly(A)+ RNA using OmniScript reverse transcriptase (OmniScript RT kit, Qiagen) with random hexamer primers.

Initial cloning of C. gibbosum and T. hamnerorum MRP fragments.

PCR and nested PCR were performed using AmpliTaq Gold DNA polymerase (Applied Biosystems) under the following conditions: 94°C for 10 min; (94°C for 15 sec, 52°C for 30 sec) for 35 cycles; 72°C for 7 min and with degenerate primers pairs (MRP_F/MRP_R and nestMRP_F/nestMRP_R) (Table 1) designed against the conserved Walker A/B domains (Allikmets & Dean 1998, Dean et al. 2001). Primers were a generous gift from David Epel and Amro Hamdoun, Hopkins Marine Station. PCR products were visualized on agarose gels, gel purified (Gene Clean II, Bio 101, Inc.), ligated into pGEM-T Easy© plasmid vector (Promega, Madison, WI), and transformed into JM109 cells (Promega). PCR products were sequenced in both directions using an ABI 3730XL capillary sequencer at the Keck facility of the Josephine Bay Paul Center for Comparative Molecular Biology & Evolution at the Marine Biological Laboratory (Woods Hole, MA). A minimum of twelve clones were sequenced for each PCR fragment. Clones were grouped based on nucleotide sequence with Sequencher 4.6 (Gene Codes Corp., Ann Arbor, MI) and a consensus sequence was generated and then examined by NCBI/GenBank BLASTx for gene identification (Altschul et al. 1997). BLASTx (NCBI) searches identified two partial sequences from *C. gibbosum* and two partial sequences from *T. hamnerorum* as having homology to MRP transporters. Gene specific primers (Table 1) were then designed to these cDNA fragments in order to obtain additional 5' and 3' sequence.

Rapid amplification of cDNA ends (RACE)

Cyphoma RACE-PCR. Poly(A)+RNA isolated from the digestive glands of snails feeding on one of four gorgonian diets (see Chapter 3, Methods) (*Briareum asbestinum, Gorgonia ventalina, Pseudopterogorgia acerosa, Pseudopterogorgia americana*) was pooled and 1 μg was primed with modified oligo (dT) primers and used to create an adaptor-ligated, double-stranded (ds) cDNA library using the Marathon cDNA Amplification Kit (BD Biosciences, Palo Alto, CA) according to the manufacturer's instructions. Gene specific primers were designed to both MRP cDNA fragments (CgMRP-1 and CgMRP-2) (Table 1) and were used in combination with adaptor-specific primers in nested 5' and 3' RACE reactions. All primers were obtained from Sigma Genosys (St. Louis, MO). Amplification of *C. gibbosum* MRP specific PCR products was carried out according to the Advantage 2 PCR Enzyme Kit (Clontech, Mountain View, CA) and cycling parameters were as follows: 94°C for 30sec; (94°C for 5 sec, 72°C for 2.5 min) for 5 cycles; (94°C for 5 sec, 70°C for 2.5 min) for 5 cycles; (94°C for 5 sec, 68°C for 2.5 min) for 25 cycles; 68°C for 5 min with the following specific primers pairs (*C. gibbosum* MRP-1, 5' RACE; MRP-R1/RACE_1_F); or 94°C for 30sec; (94°C for 5 sec, 72°C for 4 min) for 5 cycles; (94°C for 5 sec, 70°C for 4 min) for 5 cycles; (94°C for 5 sec, 68°C for 4 min) for 25 cycles with the following specific primer pairs (*C. gibbosum* MRP-1 -- CgMRP-1_F9/AP1 then CgMRP-1_F10/AP2; Cg_MRP-1_F10/AP1 then CgMRP-1_F8/AP2; CgMRP-1_R9/AP1 then CgMRP-1_R8/AP2; *C. gibbosum* MRP-2 -- CgMRP-2_F7/AP1 then CgMRP-2_F6/AP2).

The *C. gibbosum* adaptor-ligated ds cDNA library was also used to amplify partial Pgp cDNA sequences by RACE using degenerate primers designed to the conserved nucleotide binding domains. Nested-RACE was performed with the following degenerate primers pairs (Pgp_Bbox_F/RACE_1_F then Pgp_Cdomain_F/RACE_1_F) (Table 1) using the Advantage 2 PCR Enzyme Kit with the following cycling parameters: 94°C for 30sec; (94°C for 5 sec, 55°C for 30 sec, 68°C for 2 min) for 30 cycles. BLASTx searches identified the two partial sequences as having homology to Pgp. Gene specific primers were designed to both *C. gibbosum* Pgp-1 and Pgp-2 fragments and used in combination with adaptor-specific primers in nested 5' RACE reactions. PCR products were amplified with Advantage 2 polymerase with the following cycling parameters: 94°C for 30sec; (94°C for 5 sec, 72°C for 3 min) for 5 cycles; (94°C for 5 sec, 70°C for 3 min) for 5 cycles; (94°C for 5 sec, 68°C for 3 min) for 25 cycles with the following specific primer pairs (*C. gibbosum* Pgp-1 -- Pgp_R4/AP1; *C.gibbosum* Pgp-2 – Pgp_R5/AP1 then Pgp_R6/AP2) (Table 1).

Tritonia RACE PCR. One microgram of poly(A)+RNA, isolated as described above, was used to created an adaptor-ligated ds cDNA library using the Marathon cDNA Amplification Kit according to the manufacturer's instructions. Gene specific primers were designed to both MRP cDNA fragments (ThMRP-1 and ThMRP-2) (Table 1) and were used in combination with adaptor-specific primers in nested 5' and 3' RACE reactions. *T. hamnerorum* MRP specific PCR products were amplified using the Advantage 2 PCR Enzyme Kit; cycling parameters were as follows: 94°C for 30sec; (94°C for 5 sec, 72°C for 3 min) for 5 cycles; (94°C for 5 sec, 70°C for 3 min) for 5

cycles; (94°C for 5 sec, 68°C for 3 min) for 25 cycles with the following specific primer pairs (*T. hamnerorum* MRP -1 – MRP_F4/AP1 then MRP_F5/AP2; MRP_R5/AP1 then MRP_R4/AP2; ThMRP-1_R6/AP1 then ThMRP-1_R7/AP2; ThMRP-1_F7/AP1 then ThMRP-1_F6/AP1; *T. hamnerorum* MRP-2 – MRP_R3/AP1 then MRP_R2/AP2; MRP_F2/AP1 then MRP_F3/AP2; ThMRP-2_R9/AP1 then ThMRP-2_R8/AP2; ThMRP-2_F11/AP1 then MRP_F3/AP2; ThMRP-2_F10/AP1 then MRP_F3/AP2).

The *T. hamnerorum* adaptor-ligated ds cDNA library was also used to initially amplify partial Pgp cDNA sequences by RACE using a combination of degenerate primers designed to the conserved nucleotide binding region and specific adaptor primers. Nested-RACE was performed with degenerate primer pairs (Pgp_Bbox_F/RACE_1_F then Pgp_Cdomain_F/RACE_1_F) (Table 1) using the Advantage 2 PCR Enzyme Kit with the following cycling parameters: 94°C for 30sec; (94°C for 5 sec, 61°C for 30 sec, 68°C for 2 min) for 5 cycles; (94°C for 5 sec, 59°C for 30 sec, 68°C for 2 min) for 5 cycles; (94°C for 5 sec, 57°C for 30 sec, 68°C for 2 min) for 20 cycles. BLASTx searches identified the two partial sequences as having homology to Pgp. Gene specific primers were designed to both *T. hamnerorum* Pgp-1 and Pgp-2 fragments and used in combination with adaptor-specific primers in nested RACE reactions. PCR products were amplified with Advantage 2 polymerase with the following cycling parameters: 94°C for 30sec; (94°C for 5 sec, 72°C for 3 min) for 5 cycles; (94°C for 5 sec, 70°C for 3 min) for 5 cycles; (94°C for 5 sec, 68°C for 3 min) for 25 cycles with the following specific primer pairs (*T. hamnerorum* Pgp-1 – ThPgp-1_R8/AP1 then ThPgp-1_R7/AP2; ThPgp-1_R8/AP1 then ThPgp-1_R7; *T. hamnerorum*

Pgp-2 – ThPgp-2_R10/AP1 then ThPgp-2_R9/AP2) (Table 1). PCR products obtained from *C. gibbosum* and *T. hamnerorum* RACE reactions were cloned, sequenced, and analyzed as described below.

Sequence analysis

Clones were initially grouped based on nucleotide sequence identity (>80%) with Sequencher 4.6 (Gene Codes Corp., Ann Arbor, MI), and a consensus sequence was generated and examined by NCBI/GenBank BLASTx for gene identification (Altschul et al. 1997). Multiple alignments of molluscan deduced amino acid sequences were performed using ClustalW (Thompson et al. 1994). Human ABC gene subfamilies were aligned using Muscle v3.7 (Edgar 2004). In order to maintain the proper relationships between the incompletely sequenced ABC domains, *C. gibbosum* and *T. hamnerorum* Pgp and MRP sequences were aligned to each other separately and then to the human ABCB (containing Pgp) and ABCC (containing MRP) families using the profile-profile alignment settings of Muscle. Alignments were automatically masked using the alignment quality scores of Muscle; (cut-off score of 20). Maximum likelihood (ML) phylogenetic analyses were performed with RAxML (v7.0.0; Stamatakis 2006). Two types of analyses were performed: default rapid hill-climbing ML searches, starting from multiple different randomized maximum parsimony (MP) trees; or 100 replicates of rapid MP bootstrapping to find optimal start trees followed by rapid hill-climbing ML optimization. The PROTMIXWAG model of amino acid substitution was used in all analyses, which uses a category model of the WAG amino acid substitution matrix

(Whelan & Goldman 2001) during the hill-climbing ML search but a gamma distribution of substitution rates for a final tree optimization (Stamatakis 2006).

Western blot for ABC-transporters

Crude homogenates were prepared by homogenizing pooled *C. gibbosum* digestive glands and whole *T. hamnerorum* in 10 mM potassium chloride, 1.5 mM magnesium chloride, 10 mM Tris-HCl, (pH 7.4), containing 2 mM PMSF and 5% (by vol.) SDS at 4°C. In addition, livers from *Fundulus heteroclitus* collected from New Bedford Harbor, MA, were also homogenized as described and used as a positive control (Bard et al. 2002). The crude homogenate was centrifuged at 14,000 x g for 2 min at 4°C, and the supernatant was stored at -80°C until analysis. Protein concentrations were determined using the bicinchoninic acid (BCA) assay (Smith et al. 1985). Crude tissue homogenates were then diluted to 20 μg total protein with standard SDS-PAGE sample buffer (50 mM Tris, pH 6.8, 2% SDS, 10% sucrose, 0.2% β-mercaptoethanol, 0.001% bromphenol blue), denatured at 100°C for 3 min, and loaded onto a 7.5% Tris-glycine gel using a 4% stacking gel. Prestained molecular markers were purchased from Amersham (Full-range Rainbow MW Markers, GE Healthcare, Buckinghamshire, UK). Following electrophoresis, proteins were transferred onto a 0.45 μm nitrocellulose membrane (Schleicher & Schuell, Keene, NH) at 15 V for 54 min. The membrane was blocked in 5% (wt/vol) non-fat milk in Tris buffered saline-Tween 0.1% (TBST) for 1 hr and then hybridized overnight with rocking at 4°C in TBST containing 0.4 μg/mL of the monoclonal antibody C219 (Signet Laboratories, Dedham, MA). The membrane was

subsequently washed (3 x 10 min) with TBST and incubated for 1 hr. with horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Inc., West Grove, PA) diluted 1:7000 in TBST. Detection of HRP activity was by enhanced chemoluminescence (ECL) onto Hyperfilm ECL (Amersham).

Immunohistochemical analysis

Live nudibranchs and snails were transferred to a 50 mL beaker containing filtered seawater and were relaxed by adding a few crystals of magnesium sulfate until they no longer recoiled to the touch. Digestive glands and mantle tissues from *C. gibbosum* (n = 4) and whole *T. hamnerorum* individuals (n = 2) were fixed for 2 hours at 4°C in a buffered seawater solution of 2% paraformaldehyde, 2.5% glutaraldehyde (Karnovsky's Fixative) obtained from Electron Microscopy Sciences (Hatfield, PA). Tissue samples were then transferred to PBS and kept at 4°C till embedding. Paraffin-embedded samples were sectioned at 5 μm and mounted on Superfrost Plus (Fisher Scientific, St. Louis, MO) microscope slides, deparaffined, and then hydrated with normal mouse serum in 10 mM phosphate buffered saline (pH 7.5). Sections were incubated for 1 hr. at room temperature with the mAB C219 diluted (2 μg/mL) in Primary Antibody Diluent (Signet). Antibody binding was visualized with a commercial avidin-biotin-peroxidase kit using diaminobenzidine tetrahydrochlroide (DAB) as the chromogen (Vectastain® Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's instructions. Specific staining was evaluated by light microscopy by comparison of mAB C219 stained and unstained serial sections.

In-vivo dye transport assays

Fluorophores and inhibitors. MK571 was purchased from Cayman Chemical (Ann Arbor, MI). Calcein-acetoxymethylester (C-AM) was purchased from Axxora (San Diego, CA). Calcein was purchased from Invitrogen (Carlsbad, CA). Verapamil was purchased from MP Biomedicals (Solon, OH). Concentrated stocks of MK571 and C-AM were prepared in DMSO and were sufficiently concentrated so that the DMSO volume did not to exceed 0.5% of the experimental volume.

ABC transporter activity was measured by the accumulation of calcein in whole *T. hamnerorum* individuals. Calcein-AM (C-AM) is a non-fluorescent substrate for both MRP and Pgp. Any C-AM entering the cell, if not transported out immediately by either ABC transporter, is rapidly hydrolyzed by intracellular esterases to form calcein, a fluorescent membrane-impermeable molecule (Essodaigui et al. 1998). Therefore, exposure to inhibitors of ABC transporter activity results in high calcein accumulation and increased fluorescence, while reduced intracellular accumulation of calcein and fluorescence is observed when transporter activity is increased.

Nudibranchs maintained in the laboratory were removed from their host, *G. ventalina*, and allowed to depurate for four hours in a beaker of sterile-filtered seawater. Five similarly sized *T. hamnerorum* were placed in each well of a 24-well plate and incubated for two hours in 3 mL of 500 nM C-AM resuspended in sterile-filtered seawater. Incubations were performed in the presence or absence of two inhibitors at two concentrations. MK571 specifically inhibits MRP transport activity, while verapamil

inhibits Pgp transport activity. Nudibranchs were then rapidly washed two times with sterile-filtered seawater and homogenized for 30 seconds using an electric drill with a Teflon pestle in a 2 mL Wheaton glass homogenizer containing 125 µL of ethanol. The homogenate (~ 175 µL) was transferred to a 1.5 mL tube and centrifuged at room temperature for 7 min at 6.1 relative centrifugal force (RCF). Eighty-five microliters of supernatant was transferred to a 96-well black plate containing a clear bottom to minimize light scattering. The level of calcein in the resulting supernatant was measured fluorimetrically (λ_{ex} = 485 nm, λ_{em} = 530 nm) using a Cytofluor 2300 plate reader (Millepore). A calcein standard curve was run alongside the supernatant readings to estimate the concentration of intracellular calcein. The ratio of intracellular calcein fluorescence in the absence and presence of MK571 or verapamil was used as a measure of the activity of *T. hamnerorum* MRP and Pgp transporters. Statistical analysis was performed using a two sample *t* test of the means with a significance level of 5%.

Real-time quantitative RT-PCR and data analysis

Total RNA and poly(A)+ RNA (n=141) were isolated as described in Chapter 3, Methods from the digestive glands of *Cyphoma* participating in the 2006 feeding assay. Sequence-specific primers for quantitative PCR analysis of *C. gibbosum* MRP-1 mRNA expression are listed in Table 1. PCR amplification conditions, control cDNA synthesis experiments, melt curve analysis and standard curve serial dilutions specific for the representative MRP-1 clone were performed as described in Chapter 3, Methods. Each sample and standard was run in duplicate and the expression of *C. gibbosum* β-actin was

used to control for differences in cDNA synthesis among samples. Molecule number for each RNA sample was calculated from a standard curve dilution series.

To test whether *Cyphoma* MRP-1 gene expression differed between snails feeding on a control diet versus a gorgonian diet, a two way analysis of variance (ANOVA) was used with Diet (control vs. gorgonian diet) as a fixed factor and Reef (snail origin) as a random factor. The MRP-1 gene was considered a dependent variable. In addition, a one-way ANOVA was used to examine the variability in MRP-1 expression among reefs, where Reef was considered a random factor. This test was used to investigate reef-specific variation in transcript levels in time-zero snails, and to determine if any such variation persisted in snails collected from these same reefs after being fed a control diet for four days. P-values were corrected for the two-way ANOVA analysis using by Bonferroni adjustment (Sankoh et al. 1997). Data analysis was performed using SYSTAT® version 11 (Systat Software, Inc., San Jose, CA).

Results

Cloning of molluscan ABC transporter genes

An RT-PCR approach with pooled mRNA samples[1] and degenerate oligonucleotide primers were used to identify initial Pgp and MRP fragments within the conserved nucleotide binding domain of ABC transporter genes from both *Cyphoma gibbosum* and *Tritonia hamnerorum*. BlastX (NCBI) comparison of translated cDNAs indicated homology to mammalian ABCB and ABCC proteins. Initial gene fragment

[1] Molluscan mRNA samples used for the initial cloning studies were made from pooled *C. gibbosum* digestive glands and pooled *T. hamnerorum* individuals.

sizes were as follows: *C. gibbosum* MRP-1 and MRP-2, 168 bp; *C. gibbosum* Pgp-1 and Pgp-2, 216 bp; *T. hamnerorum* MRP-1 and MRP-2, 170 bp; *T. hamnerorum* Pgp-1 and Pgp-2, 71 and 182 bp respectively. Specific oligonucleotide primers were designed to these initial fragments and were used in 5' and 3' RACE reactions to obtain the eight partial MXR sequences listed in Table 2 and depicted in Figures 1 thru 10.

Sequence analysis

Deduced amino acid alignments of the molluscan MRPs and Pgps generated from nucleotide consensus sequences are shown in Figures 5 and 10. MRP amino acid alignments with human ABCC sequences revealed nearly full-length coding sequence was obtained for ThMRP-1 with only a minimal portion of the 3' end missing. The partial sequence obtained for CgMRP-1 included the putative start codon and covered the first two transmembrane spanning domains and the nucleotide binding domain. Substantially less sequence was obtained for both CgMRP-2 and ThMRP-2, which align with the first nucleotide binding domain. An alignment of molluscan Pgp amino acid sequences with mouse ABCB1a revealed that both *Tritonia* Pgp sequences align with the first nucleotide binding domain, while *Cyphoma* Pgp sequences covered the second half of the protein and included the stop codon. In addition, Kyte and Doolittle (1982) hydrophobicity plots of *C. gibbosum* and *T. hamnerorum* ABC transporters with mouse ABCB1a and human ABCC3 were nearly identical when aligned with each other (Figure 11, 12), increasing confidence in the assignment of molluscan transmembrane and nucleotide binding domains.

Phylogenetic analysis revealed homology of the molluscan ABC transporters to the human ABCB and ABCC subfamilies (Figure 13). *Tritonia* Pgp-1, Pgp-2 and *Cyphoma* Pgp-1, Pgp-2 were 63.5, 72.7, 55.5 and 55.9% identical, respectively, with overlapping portions of human ABCB1. In addition, *Tritonia* MRP-1, MRP-2 and *Cyphoma* MRP-1, MRP-2 protein sequences were 34.3, 32.2, 35.8 and 24.8% identical, respectively, with overlapping portions of human ABCC1. A second phylogenetic tree was constructed in order to better define the relationships among molluscan proteins and the human ABCB and ABCC subfamilies (Figure 14). This second tree suggested that two sets of paralogs exist for ABCB and ABCC proteins in *Tritonia* as a result of duplication events. The gene duplication resulting in ThMRP-1 and ThMRP-2 appears to have occurred prior to the *Cyphoma/Tritonia* speciation, while the duplication leading to ThPgp-1 and ThPgp-2 may have occurred after the *Cyphoma/Tritonia* speciation event. Overlapping portions of deduced amino acid sequences of *Tritonia* Pgp-1 and Pgp-2 are 67.4% identical, while *Tritonia* MRP-1 and MRP-2 are 38.4% identical. A similar comparison of *Cyphoma* proteins indicates that CgPgp-1 and CgPgp-2 (50.8% a.a. identity) arose from a more recent duplication in comparison to CgMRP-1 and CgMRP-2 (34.5% a.a. identity), which appear to be more deeply divergent, sharing a common ancestor basal to the clades containing ABCC13 and ABCC4.

Bootstrap values indicating strong support were used to assign molluscan genes to either a particular human ortholog, or when a strong relationship did not exist (bootstrap value < 80), the assignment was made to a particular clade containing multiple subfamily members. Among ABCC proteins, only human ABCC5 and CgMRP-2 are indicated as

possible orthologs; however, the bootstrap value on this pairing is very low (50%, see Figure 14), resulting in minimal confidence in this observation. Rather, CgMRP-2 should be considered an ortholog of all three human genes in this clade (ABCC5, ABCC11 and ABCC12 clade; i.e., the human genes are co-orthologs of CgMRP-2). CgMRP-1 and ThMRP-1 emerge as orthologs and together with ThMRP-2 appear to be most closely related to the human ABCC1, ABCC2, ABCC3 and ABCC6 members. Within the Pgp-related ABCB subfamily, significant bootstrap support exists for the molluscan Pgp/ABCB1/ABCB4/ABCB5/ABCB11 clade; however, more specific relationships can not be defined further because of low bootstrap support.

Expression and localization of P-glycoproteins

Expression of P-glycoprotein in *Cyphoma* digestive gland and whole *Tritonia* homogenates was examined by Western blot using the monoclonal antibody C219, which recognizes a highly conserved amino acid sequence within the nucleotide binding domain (VQ-E/A/V-ALD) (Georges et al. 1990). In *Tritonia* tissue homogenates, this antibody recognized a distinct band of expected size, 170 kDa (Juliano & Ling 1976), and two larger diffuse bands, > 200 kDa, possibility representing differentially glycosylated isoforms of Pgp (Schinkel et al. 1993) (Figure 15). However, similar immunoreactivity was absent for *Cyphoma* digestive gland homogenates. Based on deduced amino acid sequences, the lack of reactivity in *Cyphoma* homogenates is somewhat surprising considering that at least one of the *Cyphoma* proteins (CgPgp-1) contains the epitope VQEALD, among those recognized by C219. Although C219 recognizes this epitope

containg Glu (E), it does so less strongly than the epitope containing Ala (A) (VQAALD) (Georges et al. 1990). The partial *Tritonia* Pgp-1 sequence contains the Ala at this position (VQAALD), which may explain the difference in reactivity between molluscan species. Alternatively, or in addition, there may be differences in Pgp expression that contribute to the differential recognition by C219 in these immunoblot studies.

The cellular localization of P-glycoprotein was examined in paraffin-embedded fixed tissue sections probed with C219. P-glycoprotein expression was detected in the apical tips of the ciliated columnar epithelia lining the lumen of the midgut in *T. hamnerorum* individuals (Figure 16 and 17). The midgut in nudibranchs is a centrally located sac anteriorly connected to the buccal cavity containing the radula and associated feeding organs by the esophagus and posterially connected to the tubular hindgut (Millott 1937). The midgut is also connected to the caecum and the surrounding digestive gland. Heavy ciliated cells within the anterior portion of the midgut are arranged in folds and contain elongated nuclei due to the compressed condition of the cells when the midgut is not distended (Millott 1937, Morse 1968). Immunohistochemical staining helps to accentuate the folded nature of the midgut epithelia (Figure 16). Faint staining was also noted in goblet-like cells found on the nudibranch's epidermis, possibly involved in mucus secretion (Figure 16). In contrast to the staining in *Tritonia* tissues, Pgp was not detected by immunohistochemistry in *C. gibbosum* mantle or digestive gland tissues.

In vivo calcein efflux transport assay

In vivo examination of transporter activity in *Tritonia* was assessed using the calcein efflux assay. The calcein assay is a functional diagnostic method developed to quantify multixenobiotic transporter-mediated efflux activity in cells and tissues. Non-fluorescent calcein-AM rapidly traverses cell membranes where it can be converted to fluorescent calcein by non-specific esterases or extruded by Pgp and MRP before intracellular conversion to the non-MXR substrate. High levels of MXR transport activity can prevent the accumulation of free calcein in the cell, resulting in minimal fluorescent calcein accumulation. Transporter-specific MXR-mediated efflux can be measured by the use of specific MRP (MK571) and Pgp (verapamil) inhibitors. If transport activity is minimal, the transport inhibitor will have limited or no effect resulting in little increased cell fluorescence. However, if transport activity is significant, the presence of an inhibitor will increase calcein accumulation, as indicated by intracellular fluorescence, compared to incubations without the inhibitor present.

Live *Tritonia* were incubated in the presence of 500 nM extracellular C-AM with and without varying concentrations of inhibitors. The addition of verapamil or MK571 to the seawater containing *Tritonia* significantly increased calcein accumulation compared to DMSO controls (Figure 18). These results indicate that MXR-mediated efflux activity prevents calcein accumulation in *Tritonia* tissues and that inhibiting this activity causes calcein-AM to be retained and hydrolyzed to calcein. Calcein accumulation varied depending on the inhibitor used and its concentration. Increasing verapamil concentration to 25 μM did not cause a measurable increase in calcein accumulation

compared to 5 μM of verapamil, suggesting that the lower concentration of verapamil is sufficient to block the majority of Pgp transporters. In contrast, 30 μM MK571 resulted in a further 57% increase in calcein accumulation compared to that caused by 5 μM MK571, and the calcein accumulation was greater than the maximal level obtained in the presence of verapamil. This difference in the absolute level of calcein accumulation suggests that MRP transporters are either more highly expressed or more efficient at exporting C-AM in *Tritonia* tissues and therefore may play a more active role in xenobiotic transport compared to Pgp.

Quantitative RT-PCR analysis of Cyphoma MRP-1 expression

Quantitative RT-PCR analysis was used to investigate the constitutive and inducible expression of MRP-1 forms in *Cyphoma* after dietary exposure to several gorgonian species with varying allelochemical profiles. *Cyphoma* MRP-1 was selected for expression analysis because of its homology to the human MRP1 (ABCC1), known for its ability to transport a range of glutathione-conjugated substrates. This is particularly relevant to the present study because *Cyphoma* digestive gland tissues constitutively express high levels of the glutathione-conjugating enzyme, glutathione *S*-transferase, likely necessitated by the presence of numerous potent inhibitors/substrates in its gorgonian diets (see Chapter 4). Thus, efflux of glutathione-conjugated allelochemicals from *Cyphoma* digestive gland may be mediated by MRP-1.

Adult *Cyphoma* were allowed to feed ad libitum for 4 d on one of seven gorgonian diets or a control diet devoid of gorgonian allelochemicals, as described in

Chapter 3. Digestive gland levels of CgMRP-1 transcripts were measured by quantitative RT-PCR. MRP-1 transcripts were constitutively expressed in *Cyphoma* digestive gland and the expression was not affected by gorgonian diet (Table 3, Figure 19). MRP-1 transcript expression was also measured in time-zero individuals to determine if collection location could influence gene expression. MRP-1 expression was found to significantly differ in time-zero snails as a function of reef location (ANOVA, $F_{4,26} = 2.74$, $p = 0.05$, Table 4, Figure 20), but did not significantly differ among snails allowed to feed on a control diet for four days (ANOVA, $F_{4,28} = 0.637$, $p = 0.640$).

Discussion

In aquatic systems, much of our knowledge of the function of ABC transporters in marine invertebrates is limited to the response of these proteins under anthropogenic stress conditions (Kurelec 1992, Minier et al. 1993, Smital et al. 2000, Smital et al. 2003, Kingtong et al. 2007). However, for marine consumers that regularly feed on prey/hosts laden with natural toxins, efflux transporters may play a critical role in regulating the absorption of allelochemicals, thereby dictating diet selection. This study is the first to examine the expression and activity of ABC transporters from two molluscan predators that feed solely on chemically defended prey.

Molluscan P-glycoproteins

Using a combination of molecular approaches, four partial Pgps related to multixenobiotic transporters in vertebrates were identified from the digestive gland of

Cyphoma gibbosum (CgPgp-1, CgPgp-2) and whole *Tritonia hamnerorum* (ThPgp-1, ThPgp-2). All four molluscan Pgps encode transporters that cluster within a highly supported clade containing the human ABCB1, ABCB4, ABCB5 and ABCB11 genes. ABCB1 (MDR1 or Pgp1) is possibly the most well studied of all the multixenobiotic transporters and is known for its promiscuous transport of hydrophobic substrates, including xenobiotics, lipids, sterols, and chemotherapeutics derived from natural products (Choudhuri & Klaassen 2006). ABCB4 (MDR3) and ABCB11 are both expressed in the liver and are involved in the secretion of bile acids (Gerloff et al. 1998, Oude Elferink & Paulusma 2007), while ABCB5 is hypothesized to function as a drug resistance mediator, similar to ABCB1, and is primarily expressed in the epidermis (Frank et al. 2005). Acquisition of complete ABCB sequences would no doubt help to better define the relationships of the molluscan Pgps with particular ABCB members. Nevertheless, the phylogenetic analysis presented here clearly groups these molluscan transporters with those ABCB members known to mediate xenobiotic resistance.

Western blot analysis of molluscan tissues with the mAb C219, which recognizes both human MDR1 (ABCB1) and MDR3 (ABCB4) proteins (Van den Elsen et al. 1999), suggested that homologs of human MDR proteins are expressed in *Tritonia* tissues but not in *Cyphoma* digestive gland. The disparity in Pgp expression between both molluscan predators may reflect differences in their foraging behavior. Specialist herbivores that consume a diet rich in allelochemicals had a higher intestinal Pgp capacity in comparison to a sympatric generalists that are unable to tolerate the toxin-laden diet (Green et al. 2004). While high Pgp protein expression does not necessarily

translate to increased protein activity, these results do suggest that dietary toxins could be handled differently depending on consumer experience.

Deduced amino acid sequences from two partial *Cyphoma* Pgps indicate that at least one of the forms (CgPgp-1) contains the correct epitope (VQEALD) in its nucleotide binding domain and therefore would be recognized by C219 if expressed in the digestive gland. It is possible that CgPgp-1 is expressed in tissues other than the digestive gland; however, immunohistochemical analysis of whole *Cyphoma* sections with C219 did not detect any immunoreactive protein. Based on sequence data, multiple forms of Pgp are expressed in *Cyphoma* digestive gland, yet their detection may be limited by the reactivity of the antibody used here, suggesting that western blots should be interpreted with caution. In contrast, *Tritonia* tissue homogenates displayed a distinct band at 170 kDa, in agreement with human MDR proteins (Choudhuri & Klaassen 2006) and two diffuse bands at > 200 kDa. Similar multiple banding patterns (i.e., 170 kDa, and > 200 kDa banding) have also been observed in the gill tissue of mussels *Mytilus californianus*, *M. galloprovincialis*, and *M. edulis* and oysters *Crassostrea gigas* and *C. virginica* (i.e., > 200 kDa) (reviewed in Bard 2000). Protein bands > 200 kDa may represent post-translational modifications or differential glycosylation states, not uncommon among mammalian P-glycoproteins (Schinkel et al. 1993).

Immunohistochemical staining of *Tritonia* tissues by C219 indicated that Pgp proteins were localized to the apical epithelial of the midgut and to a lesser extent in the epidermis. The location of Pgp in *Tritonia* digestive tissues is in agreement with the localization of mammalian ABCB1 on the apical (or luminal) surface of polarized

epithelia of many tissues, such as the gastrointestinal tract, kidney proximal tubules and biliary heptocytes (Klein et al. 1999). Based on the location of mammalian ABCB1 in barrier tissues coupled with its function as a unidirectional transporter of a range of toxic substrates, it is likely that the physiological role of ABCB1 and its orthologs is to protect cells and ultimately organisms against toxic compounds (Schinkel et al. 1994, Schinkel et al. 1997). The two tissues types observed to exhibit the greatest Pgp expression in *Tritonia*, the gut epithelia and the epidermis, correspond to those tissues that would be exposed to the greatest concentration of gorgonian allelochemicals, due to the fact that *Tritonia* both resides on and consumes its gorgonian host.

Interestingly, this nudibranch is able to selectively sequester a furano-germacrene feeding deterrent from its gorgonian host, *Gorgonia ventalina*, and concentrate this chemical defense four-fold (dry mass basis) relative to the gorgonian (Cronin et al. 1995). While the exact mechanism of sequestration in nudibranch tissues is unknown, recent studies have demonstrated that mammalian MXR transporters can reduce the intracellular concentration of toxins by actively sequestering them in subcellular compartments, away from vulnerable cellular targets (Van Luyn et al. 1998, Molinari et al. 2002, Rajagopal & Simon 2003, Ifergan et al. 2005). Furthermore, MXR members can have different activity profiles depending on membrane location (i.e., plasma membrane versus lysosomal membrane), which may be linked to the lipid environment or post-translational modification of the transporter that can occur within subcellular compartments (Rajagopal & Simon 2003). This intracellular sequestration phenomenon has also been observed in mussel blood cells, whereby the intracellular accumulation of the fluorescent

Pgp substrate Rhodamine B in lysosomes could be reversed if incubated with verapamil (Svensson et al. 2003). In addition, the MXR-mediated subcellular localization is suspected to be responsible for the sequestration of the marine toxin, okadaic acid in the mussel *Mytilus edulis* (Svensson et al. 2003). Transporters have also been suggested as a mechanism by which herbivorous insects can sequester unmetabolized host plant toxins in diverticular pouches, defensive glands or hemolymph (Sorensen & Dearing 2006). Therefore, sequestration of dietary allelochemicals by ABC transporters may function as a protective mechanism against cytotoxicity, with the added advantage of providing the marine consumer with its own chemical protection.

The *in vivo* activity of MXR transporters was investigated in *Tritonia* using ABC subfamily specific transport inhibitors capable of blocking the Pgp- and MRP-mediated efflux of C-AM. Both inhibitors resulted in increased dye accumulation in *Tritonia* tissues, which is consistent with previous competitive dye transport assays reporting MXR-mediated transport activity in a variety of marine invertebrates (grass shrimp (Finley et al. 1998), worm (Toomey & Epel 1993), sponge (Muller et al. 1996), oyster (Keppler 1997), mussel (McFadzen et al. 2000), sea urchin (Hamdoun et al. 2004)). Varying inhibitor concentration did not increase Pgp-mediated calcein accumulation, but did cause a substantial impact on MRP-mediated calcein accumulation. The greater level of calcein accumulation in *Tritonia* exposed to MRP inhibitor suggests that MRP-mediated efflux may contribute more to xenobiotic resistance than Pgp in *Tritonia*.

Molluscan multixenobiotic resistance-associated proteins

While both subfamilies of transporters confer multixenobiotic resistance, the substrate selectivities of Pgp and MRP differ markedly. Pgp transport neutral or mildly positive lipophilic compounds, while MRP substrates are lipophilic anions capable of transporting a range of substrates including glutathione (GSH), glucuronide, or sulfate conjugates of phase II detoxification reactions (Kruh & Belinsky 2003). Phylogenetic analysis of molluscan MRP sequences obtained here indicate that CgMRP-1, ThMRP-1 and ThMRP-2 fall within a well-supported clade containing the mammalian ABCC1, ABCC2, ABCC3 and ABCC6 proteins, known for their ability to transport a variety of glutathione conjugates including natural product cancer drugs and prostaglandins (Evers et al. 1997, de Waart et al. 2006). The Caribbean gorgonian *Plexaura homomalla* is regularly consumed by *Cyphoma* despite the high concentration of prostaglandin A_2 esters (Gerhart 1986), which function as potent feeding deterrents in this coral (Gerhart 1984, Pawlik & Fenical 1989). Recent evidence suggests that *Cyphoma* may be able to tolerate dietary prostaglandins and other lipophilic gorgonian compounds by conjugation with glutathione *S*-transferases (GSTs). GSTs are highly expressed in the digestive gland of this predator, and a screening of gorgonian lipophilic extracts suggests all gorgonian diets may contain substrates for *Cyphoma* GSTs (Chapter 4). Therefore, the constitutive expression of MRP-1 in the digestive gland of *Cyphoma* may facilitate the efficient biliary excretion of putative glutathione-conjugates of prostaglandins and other lipophilic compounds (Evers et al. 1997, Paumi et al. 2003, de Waart et al. 2006). MRP-1 expression was also detected in the snails feeding on control diets devoid of gorgonian

allelochemicals, suggesting that these transporters may also be responsible for the export of physiological substrates, in addition to their roles in multixenobiotic resistance. In contrast, MRP-1 expression significantly varied in time-zero snails as a function of reef location. While it is difficult to conclusively pin point the cause of this reef-specific variation, the length of snail foraging time on a single colony or the presence of additional gorgonian species not tested in our feeding assays may have had an effect on MRP-1 expression.

Phylogenetic analysis revealed that *Cyphoma* MRP-2 is significantly divergent from the other molluscan MRPs identified in this study and falls into the clade containing human ABCC5, ABCC11 and ABCC12. All three of these transporters are at the early stages of investigation and their physiological functions are unknown, yet studies indicate that they mediate the transport of antiretroviral nucleosides and lipophilic glutathione-conjugates (Wijnholds et al. 2000, Kruh et al. 2007).

Mounting evidence suggests that ABC transporters likely regulate the absorption and subsequent distribution of natural toxins in marine organisms (Toomey & Epel 1993, Toomey et al. 1996, Keppler & Ringwood 2001, Eufemia et al. 2002). The present work describes the first efforts to identify MXR genes that may protect marine invertebrates that have adapted to exclusively feed on allelochemically-rich prey. Messenger RNA encoding MXR transporters is expressed in both generalist and specialist molluscan species and share sequence and organizational structure similar to MXRs of distantly related organisms, indicating the likelihood of shared function as natural product transporters between mammalian and molluscan orthologs. A second piece of evidence

supporting the role of MXR proteins as dietary allelochemical efflux pumps comes from the results of immunohistochemical analyses in *Tritonia* tissues, where Pgp expression was highest in the tissues most vulnerable to allelochemical exposure. Additionally, *in vivo* studies of MXR activity using ABC subfamily-specific inhibitors provided added support for the presence and function of MXR transporters in *Tritonia*. However, for organisms where *in vivo* dye assays may not be permitted, real-time qPCR provided a highly robust and sensitive method for quantifying MXR isoform-specific expression. Constitutive expression of selected promiscuous MXR transporters may be advantageous to a generalist predator with a chemically diverse diet. Support for this hypothesis may come from screening gorgonian extracts in competitive substrate inhibition assays with transfected cell lines or isolated membrane vesicles overexpressing molluscan MXRs of interest. This initial screening approach would facilitate comparisons of substrate breadth between MXRs from generalist and specialist consumers and allow one to begin to test predictions about whether generalist MXRs possess greater structural and functional flexibility in their substrate specificity compared to specialists (Li et al. 2004).

In summary, the elucidation of the molecular mechanisms underlying consumer tolerance is a prerequisite to understanding the foraging behavior of marine consumers. This study provides a much needed first step in identifying putative environmental chemical stress genes capable of ameliorating allelochemical-induced toxicity. It is likely that both MRP and Pgp play significant roles in natural toxicant efflux in marine consumers. Future work aimed at elucidating those marine natural products capable of

interacting with substrate-binding sites on MXRs will provide valuable insight into the evolution of ABC transporter-mediated consumer counter-defense mechanisms.

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Table 1. Primers used in the present study

Gene	Primers	Direction	Sequences (5' to 3')
Degenerate			
MRP	MRP_F	Forward	CTD GTD GCD GTD GTD GG
	MRP_R	Reverse	RCT NAV NGC NSW NAR NGG NTC RCT
	nest_MRP_F	Forward	CGG GAT CCA GRG ARA AYA THC TNT TTG G
	nest_MRP_R	Reverse	CGG AAT TCN TCR TCH AGN AGR TAD ATR TC
P-gp	Pgp_Bbox_F	Forward	GGI GGI CAG AAR CAR MGI ATI GC
	Pgp_Cdomain_F	Forward	GAY GAR GCI ACI TCI GCI CTI G
Specific			
Cyphoma MRP-1	MRP-R1	Reverse	CCG GGC TAA ACT CAC TCT CTG TTT CTG G
	CgMRP-1_R8	Reverse	CAT CCA TGA CCT GAG AAG ATC TGG
	CgMRP-1_R9	Reverse	GGT TCA CAA ACT GCA ACA GGT CG
	CgMRP-1_F8	Forward	CCA GAA ACA GAG AGT GAG TTT AGC C
	CgMRP-1_F9	Forward	GAA TGG CAC CGT CAT CGT ACA GAG
	CgMRP-1_F10	Forward	ATT GAC GCC TGT GCC CTT CGC AC
Cyphoma MRP-2	CgMRP-2_F6	Forward	GAG ACA TTT GGA GCT GGG GAC CAG
	CgMRP-2_F7	Forward	GTG TCA GCA TGC TGT CTG GAC AAA G
Cyphoma Pgp-1	P-gp_R4	Reverse	CCT CTG TCA CCA CAC CAT GTC G
Cyphoma Pgp-2	P-gp_R5	Reverse	GTT GGC CAC AAA GCC AGC GTA GGC
	P-gp_R6	Reverse	CTC CAA CAC CTG ACC GTT CTC C
Tritonia MRP-1	MRP_F4	Forward	CAA TCG GAT ATT GAC ATT CTC CCT GGC
	MRP_F5	Forward	GCT GTG TAC AGT GAC CAA GAC
	MRP_R4	Reverse	CAG GGA GAA TGT CAA TAT CCG ATT GG
	MRP_R5	Reverse	GTC TTG GTC ACT GTA CAC AGC
	ThMRP-1_F6	Forward	AAA TGA CGT CCG CTG CTG GTG TCC
	ThMRP-1_F7	Forward	AGG CAT TTG GCT GAC GGA GTG GAC
	ThMRP-1_R6	Reverse	AAA CAT GGC CAC GCC TAG TTG GTC
	ThMRP-1_R7	Reverse	GGA AGT CTC ATC GCC AAT GCT GG
Tritonia MRP-2	MRP_F2	Forward	GGT CCA GAT CTG CAA ATG CTG
	MRP_F3	Forward	GCA AAT GCT GCC AGA TGG TGA TC
	MRP_R2	Reverse	CAG CAT TTG CAG ATC TGG ACC
	MRP_R3	Reverse	GAT CAC CAT CTG GCA GCA TTT GC
	ThMRP-2_R9	Reverse	GGA CTT GAC CAA ATT GCC CAG CC
	ThMRP-2_R8	Reverse	TGA CCC GTG AAG TGA AGC GCT ATC
	ThMRP-2_F10	Forward	GTG GAA GGG AGT GTT GTC ATG AGG
	ThMRP-2_F11	Forward	GAA TCA ACG TGA CGA TTC CAG ACC
Adaptor primers	RACE_1_F		AAT ACG ACT CAC TAT AGG
	AP1		CCA TCC TAA TAC GAC TCA CTA TAG GGC
	AP2		ACT CAC TAT AGG GCT CGA GCG GC
Cyphoma MRP-1 qPCR primers	136_1_F1	Forward	ACG AGG AGC TGA TGT GTC ACG ATG G
	136_1_R1	Reverse	GTC CTC CTC ATC CAC ATC TTC ATC G

Table 2. Summary of ABC transporter cDNAs obtained from *Cyphoma gibbosum* and *Tritonia hamnerorum*

Subfamily	Species	Tissue	Gene designation	Length of fragment		GenBank Acc. #
				Nucelotide (bp)	Amino acid (a.a.)	
ABCB	*Cyphoma gibbosum*	digestive gland	Pgp-1	1755	584	EU487190
			Pgp-2	1029	342	EU487191
	Tritonia hamnerorum	whole animal	Pgp-1	390	130	EU487196
			Pgp-2	410	136	EU487197
ABCC	*Cyphoma gibbosum*	digestive gland	MRP-1	2853	951	EU487192
			MRP-2	554	184	EU487193
	Tritonia hamnerorum	whole animal	MRP-1	4647	1549	EU487194
			MRP-2	2297	765	EU487195

Table 3. Results of a two-way ANOVA investigating differences in MRP-1 gene expression in *C. gibbosum* feeding on control versus gorgonian diets.

Factors	df	MS	*F*	p
Control vs. *B. asbestinum*				
Diet	1	$5.3x10^{9}$	0.797	0.422
Reef	4	$1.4x10^{10}$	0.423	0.791
Diet x Reef	4	$6.6x10^{9}$	0.204	0.935
Error	36	$3.2x10^{10}$		
Control vs. *E. mammosa*				
Diet	1	$5.4x10^{9}$	3.22	0.147
Reef	4	$3.2x10^{9}$	0.106	0.980
Diet x Reef	4	$1.7x10^{10}$	0.555	0.697
Error	35	$3.0x10^{10}$		
Control vs. *G. ventalina*				
Diet	1	$5.9x10^{10}$	5.91	0.072
Reef	4	$1.4x10^{10}$	0.432	0.785
Diet x Reef	4	$1.0x10^{10}$	0.313	0.867
Error	35	$3.2x10^{10}$		
Control vs. *P. acerosa*				
Diet	1	$2.0x10^{10}$	0.948	0.385
Reef	4	$5.5x10^{10}$	1.87	0.139
Diet x Reef	4	$2.1x10^{10}$	0.723	0.582
Error	33	$3.0x10^{10}$		
Control vs. *P. americana*				
Diet	1	$1.9x10^{10}$	1.01	0.371
Reef	4	$2.5x10^{10}$	0.852	0.502
Diet x Reef	4	$1.9x10^{10}$	0.651	0.629
Error	35	$2.9x10^{10}$		
Control vs. *P. elisabethae*				
Diet	1	$1.7x10^{10}$	5.02	0.154
Reef	2	$1.8x10^{10}$	1.30	0.297
Diet x Reef	2	$3.5x10^{10}$	0.249	0.782
Error	18	$1.4x10^{10}$		
Control vs. *P. homomalla*				
Diet	1	$6.4x10^{10}$	9.66	0.036
Reef	4	$4.5x10^{9}$	0.157	0.958
Diet x Reef	4	$6.6x10^{9}$	0.230	0.920
Error	34	$2.9x10^{10}$		

A $p \leq 0.007$ should be accepted as significant (Bonferroni adjusted).

Table 4. Results of a one-way ANOVA investigating reef-dependent MRP-1 gene expression in time-zero and four day control fed *C. gibbosum*. Reefs considered random factors.

Factors	df	MS	F	p
Reef (Experiment 1)				
Control diet	4, 28	2.1×10^{10}	0.637	0.640
Reef (Experiment 2)				
Time-zero group	4, 26	3.1×10^{10}	2.74	**0.050**

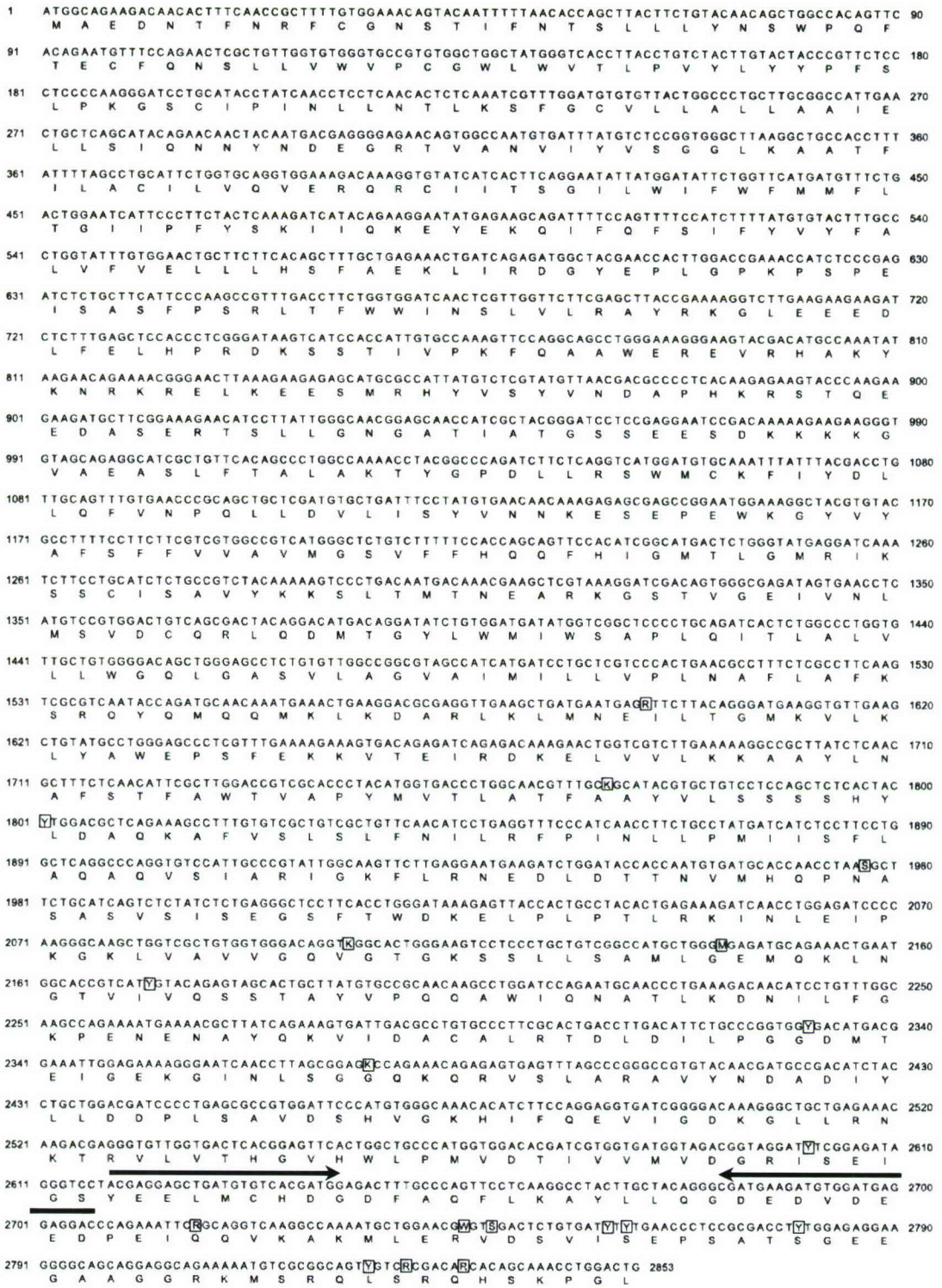
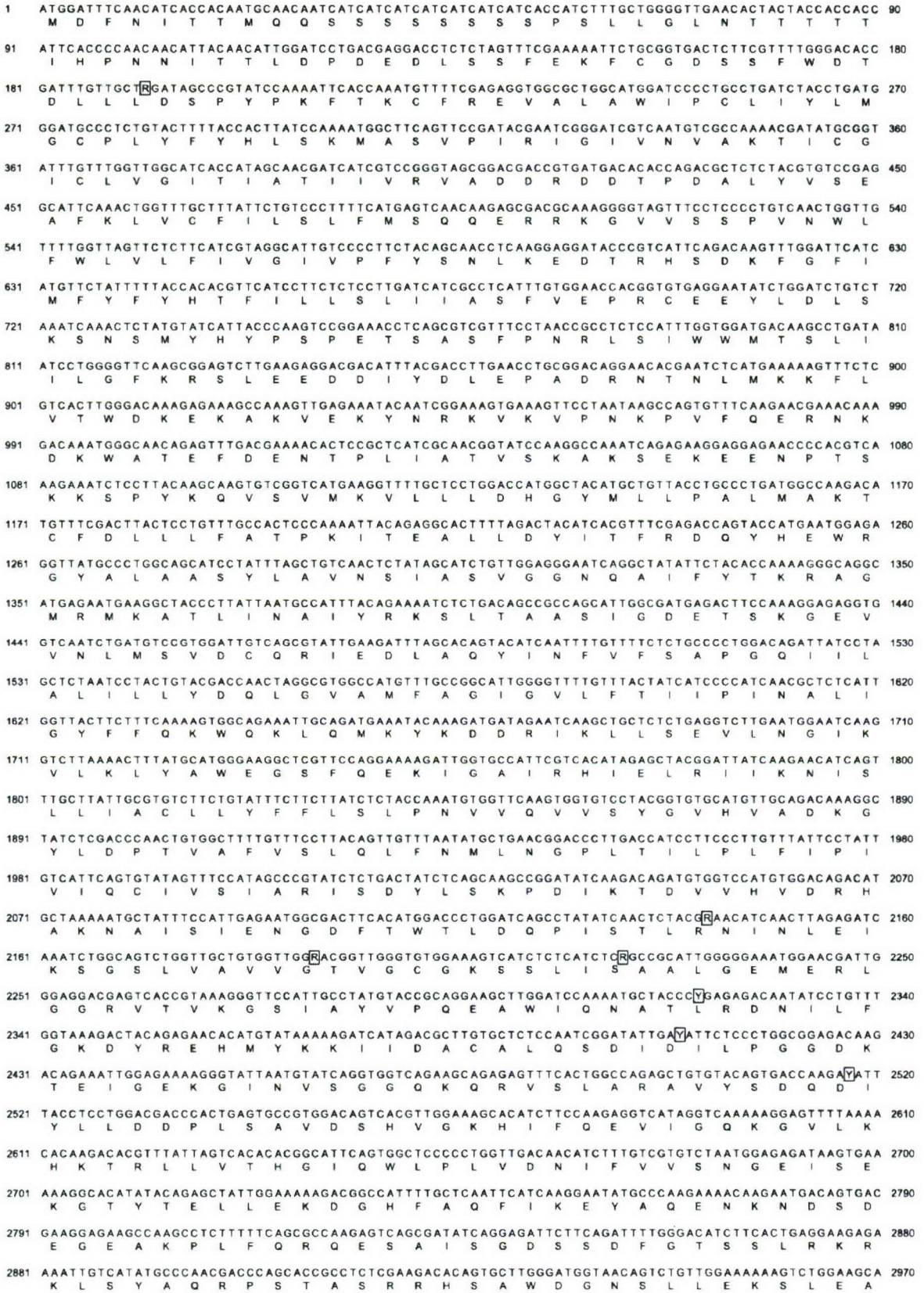


Figure 1. Partial nucleotide and deduced amino acid sequence of *Cyphoma* MRP-1 cDNA. The *Cyphoma* MRP-1 nucleotide polymorphisms (boxed) are indicated according to IUPAC naming conventions. Arrows indicate location of quantitative PCR primers. GenBank Accession number EU487192.



Figure 2. Partial nucleotide and deduced amino acid sequence of *Cyphoma* MRP-2 cDNA. The *Cyphoma* MRP-2 nucleotide polymorphisms (boxed) are indicated according to IUPAC naming conventions. GenBank Accession number EU487193.



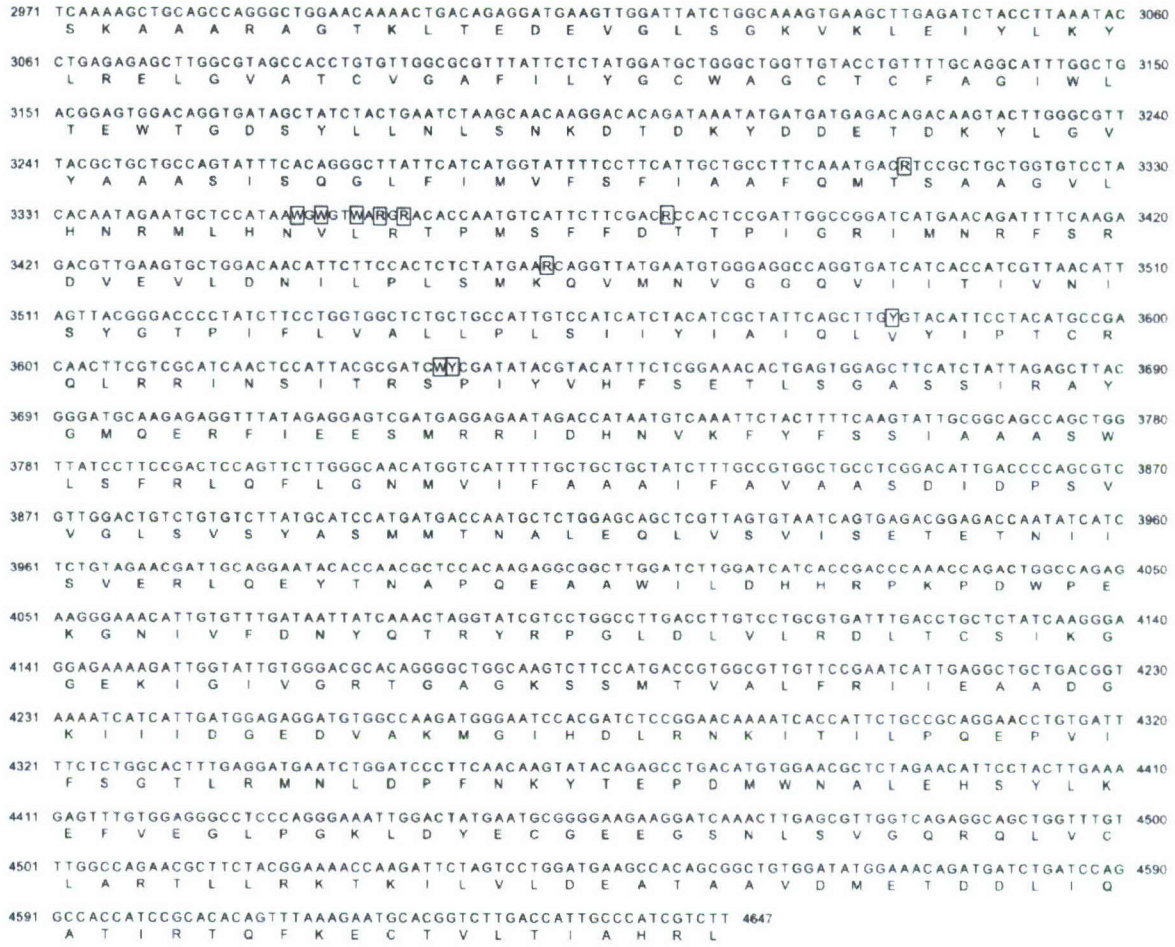


Figure 3. Partial nucleotide and deduced amino acid sequence of *Tritonia* MRP-1 cDNA. The *Tritonia* MRP-1 nucleotide polymorphisms (boxed) are indicated according to IUPAC naming conventions. GenBank Accession number EU487194.

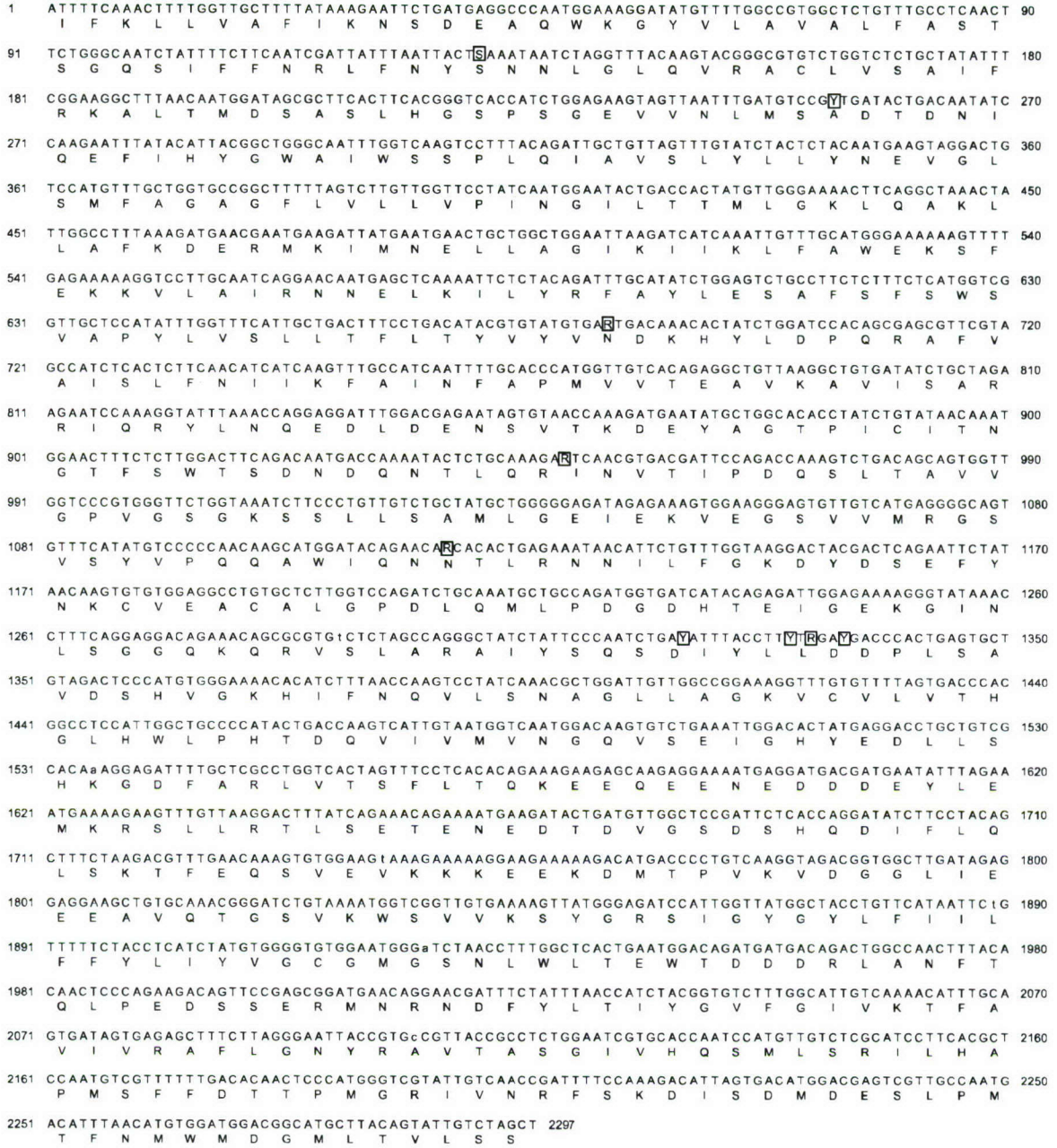


Figure 4. Partial nucleotide and deduced amino acid sequence of *Tritonia* MRP-2 cDNA. The *Tritonia* MRP-2 nucleotide polymorphisms (boxed) are indicated according to IUPAC naming conventions. GenBank Accession number EU487195.

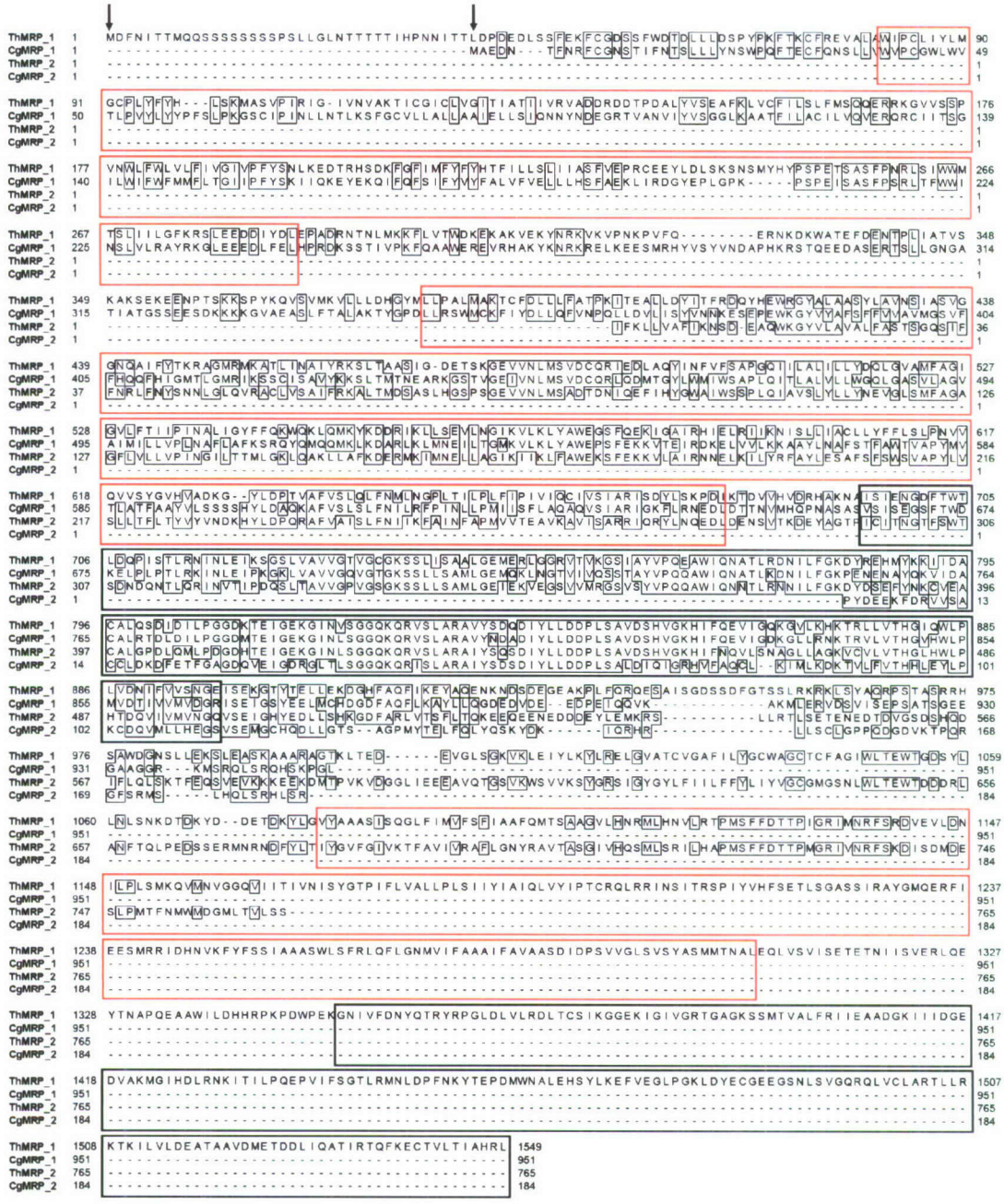


Figure 5. Alignment of deduced amino acid sequences of *Cyphoma* and *Tritonia* MRP proteins. Molluscan deduced amino acids sequences were aligned using ClustalX. Identical residues in at least two sequences are shaded in grey. Black and red boxes represent the putative nucleotide binding domains and hydrophobic transmembrane domains, respectively. Domains were predicted using the algorithm of Kyte and Doolittle (1982) (window of 13 amino acids) and with NCBI Conserved Domain Database searches (Marchler-Bauer 2007). Predicted start codons for *Cyphoma* MRP-1 and *Tritonia* MRP-1 are indicated by a downward arrow above the alignment. A dash (-) indicates missing or gapped residues. GenBank sequences in the alignment include: *Cyphoma* MRP-1 (EU487192); *Cyphoma* MRP-2 (EU487193); *Tritonia* MRP-1 (EU487194); *Tritonia* MRP-2 (EU487195).

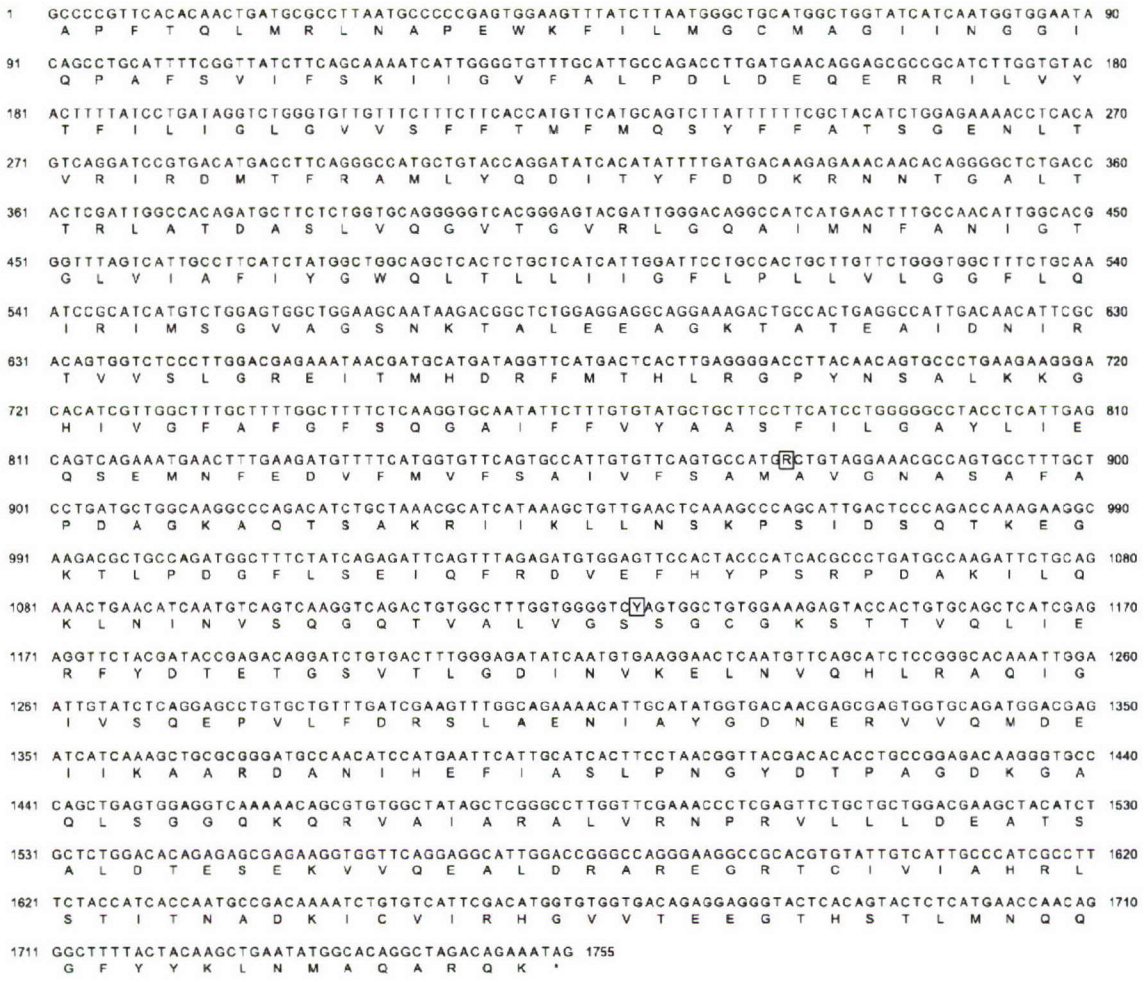


Figure 6. Partial nucleotide and deduced amino acid sequence of *Cyphoma* Pgp-1 cDNA. The *Cyphoma* Pgp-1 nucleotide polymorphisms (boxed) are indicated according to IUPAC naming conventions. The stop codon is indicated by an asterisk. GenBank Accession number EU487190.

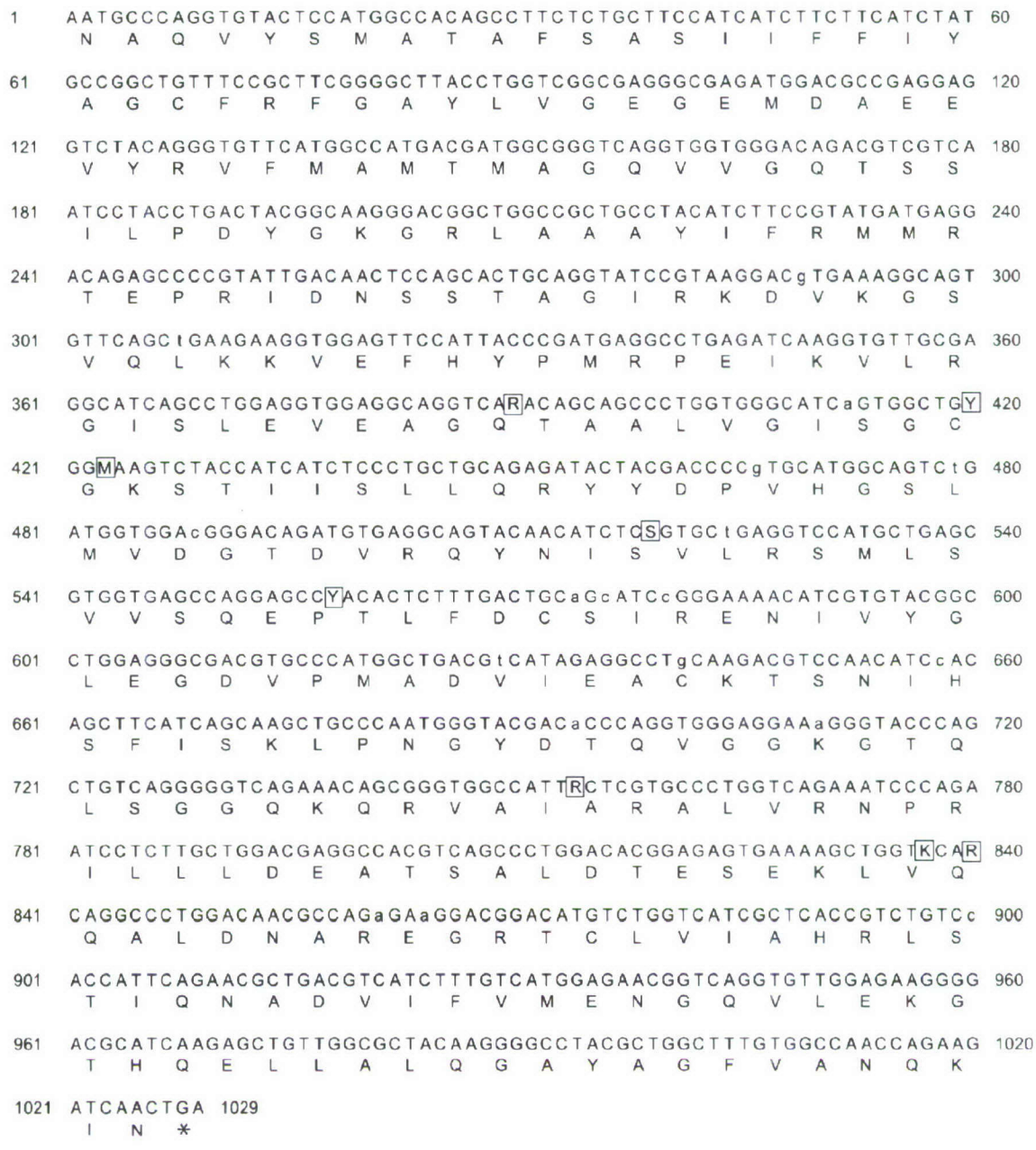


Figure 7. Partial nucleotide and deduced amino acid sequence of *Cyphoma* Pgp-2 cDNA. The *Cyphoma* Pgp-2 nucleotide polymorphisms (boxed) are indicated according to IUPAC naming conventions. The stop codon is indicated by an asterisk. GenBank Accession number EU487191.



Figure 8. Partial nucleotide and deduced amino acid sequence of *Tritonia* Pgp-1 cDNA. The *Tritonia* Pgp-1 nucleotide polymorphisms (boxed) are indicated according to IUPAC naming conventions. GenBank Accession number EU487196.



Figure 9. Partial nucleotide and deduced amino acid sequence of *Tritonia* Pgp-2 cDNA. The *Tritonia* Pgp-2 nucleotide polymorphisms (boxed) are indicated according to IUPAC naming conventions. GenBank Accession number EU487197.

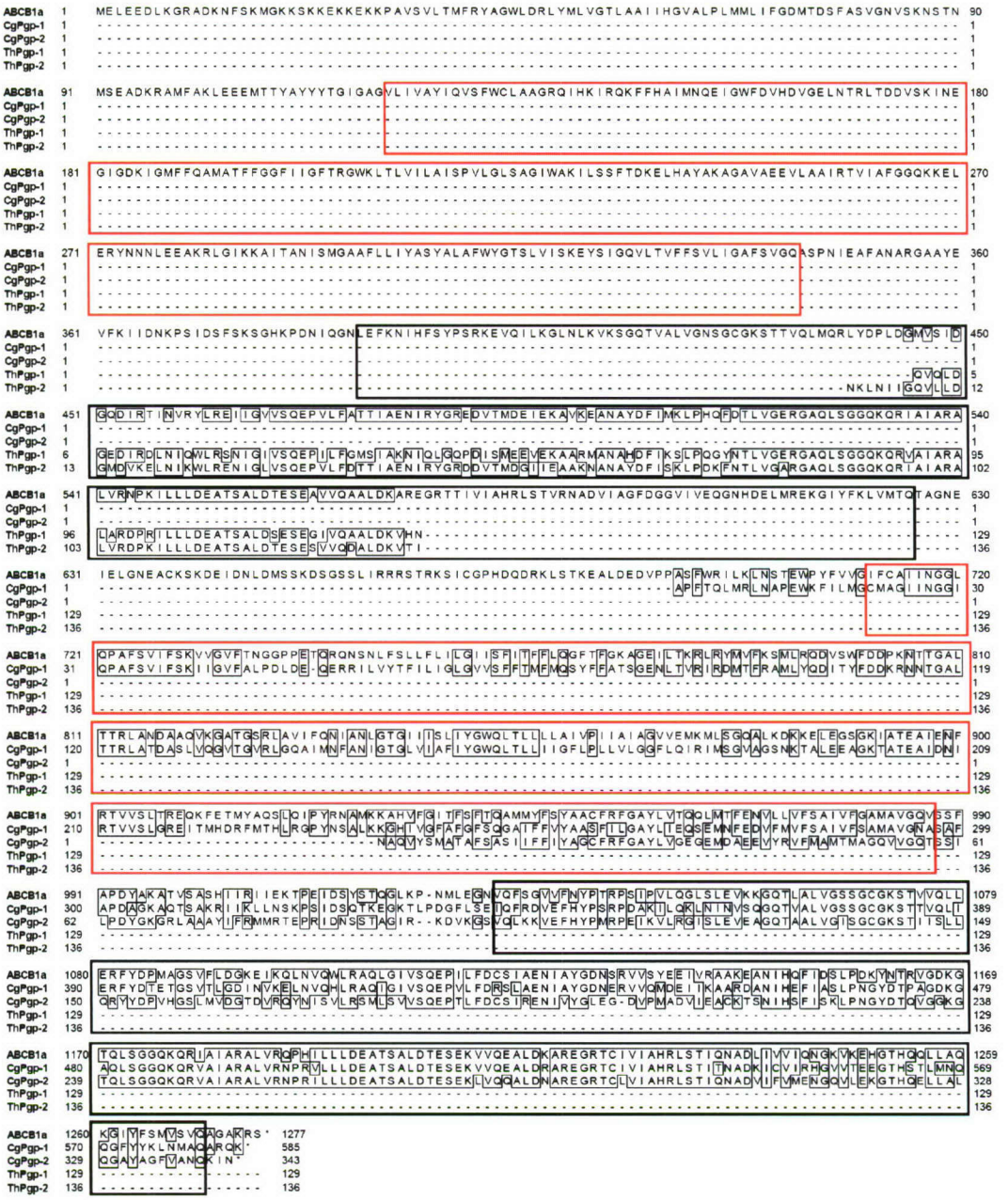


Figure 10. Alignment of deduced amino acid sequences of *Cyphoma* and *Tritonia* Pgp proteins with Mouse ABCB1a. Molluscan deduced amino acid sequences and mouse protein sequence were aligned using ClustalX. Identical residues are shaded in grey. Black and red boxes represent the putative nucleotide binding domains and hydrophobic transmembrane domains, respectively. Transmembrane and nucleotide binding domains were predicted using the algorithm of Kyte and Doolittle (1982) (window of 13 amino acids) and with NCBI Conserved Domain Database searches (Marchler-Bauer et al. 2007). An asterisk indicates a stop codon and a dash (-) indicates missing or gapped residues. GenBank sequences in the alignment include: mouse ABCB1a (*M. musculus*, NP_035206), *C. gibbosum* Pgp-1 (EU487190), *C. gibbosum* Pgp-2 (EU487191), *T. hamnerorum* Pgp-1 (EU487196), *T. hamnerorum* Pgp-2 (EU487197).

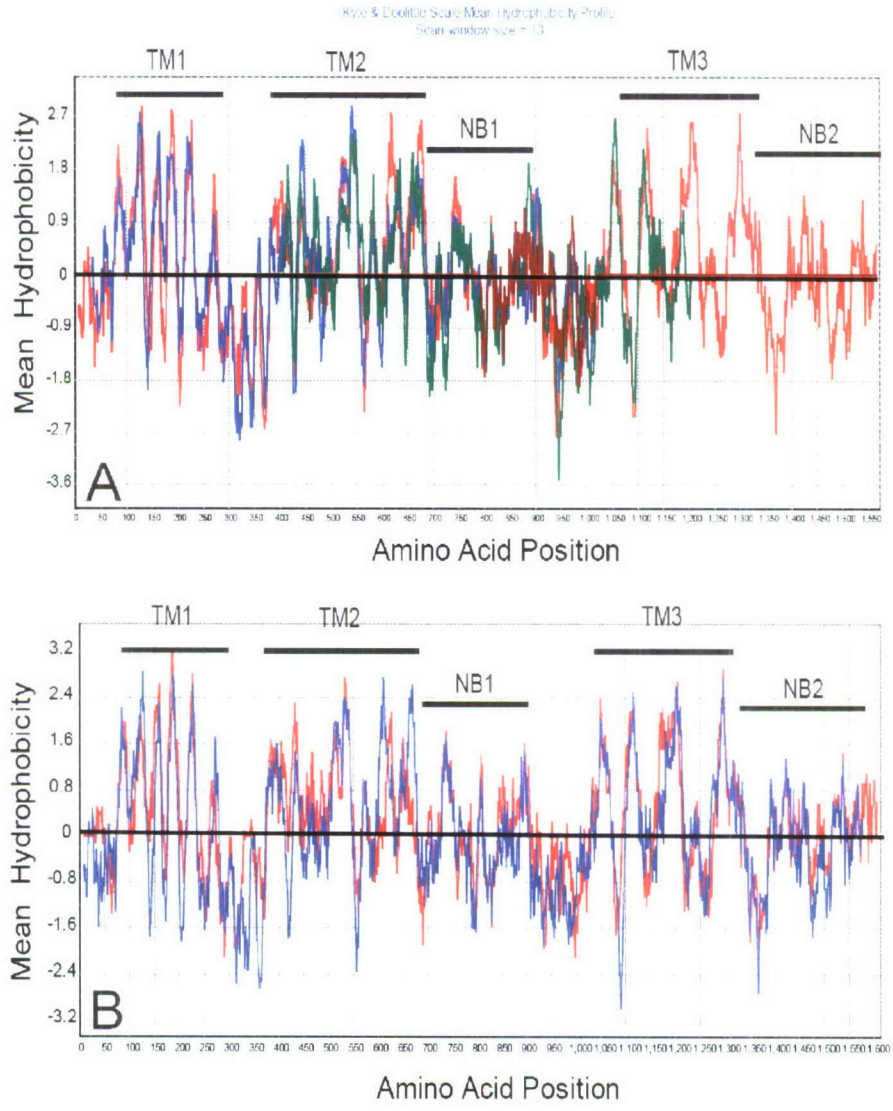


Figure 11. Comparison of molluscan and human MRP hydropathy profiles. (**A**) Deduced amino acid sequences from *Cyphoma* and *Tritonia* MRP profiles were aligned using ClustalX. *Tritonia* MRP-1 plot is indicated by the red line and the *Cyphoma* MRP-1 (blue line), *Tritonia* MRP-2 (green line) and *Cyphoma* MRP-2 (maroon line) are layered on top. (**B**) Deduced amino acid sequence from *Tritonia* MRP-1 (blue line) was aligned with human ABCC3 (red line) using ClustalX. Black bars above the plots indicate positions of the three transmembrane regions (TM1, TM2, TM3) and nucleotide binding domains (NB1, NB2) in ABCC proteins. Values above and below the horizontal line indicate hydrophobic and hydrophilic regions, respectively. Hydrophobicity plots were generated using the Kyte-Doolittle algorithm (1982) with a window of 13 residues by BioEdit v7.0.5.2. GenBank sequences in the alignment include: human ABCC3 (NP_003777.2), *C. gibbosum* MRP-1 (EU487192), *C. gibbosum* MRP-2 (EU487193), *T. hamnerorum* MRP-1 (EU487194), *T. hamnerorum* MRP-2 (EU487195).

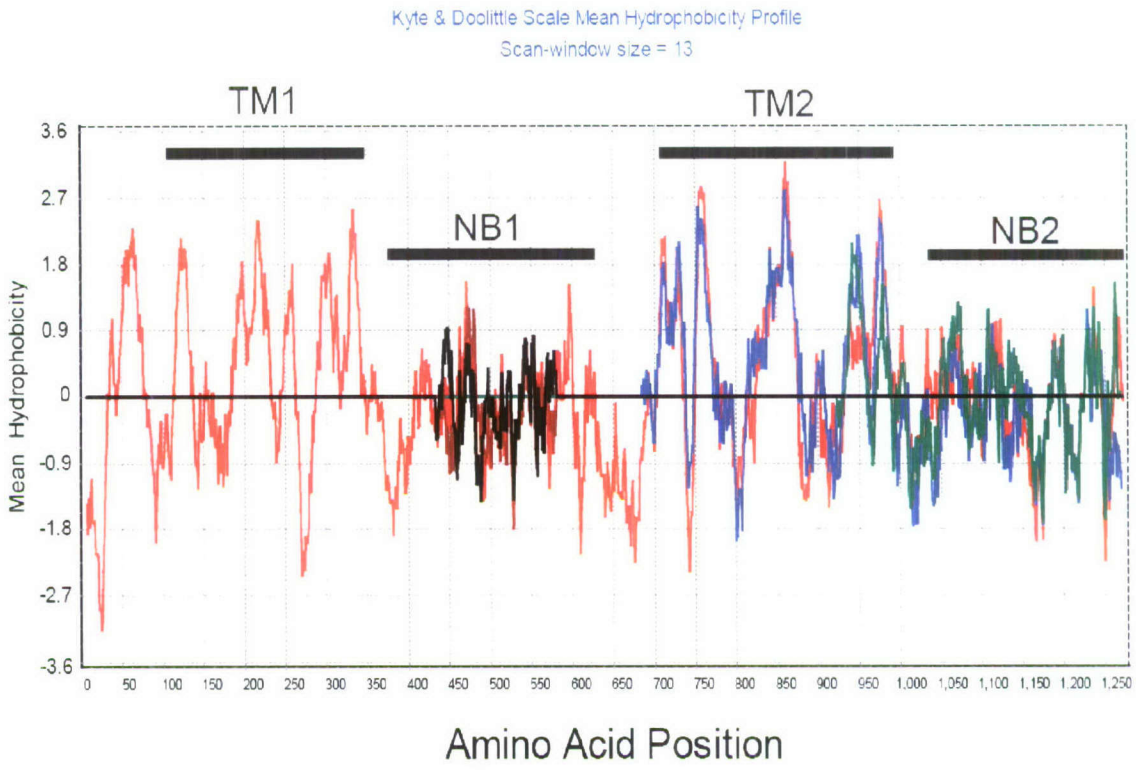
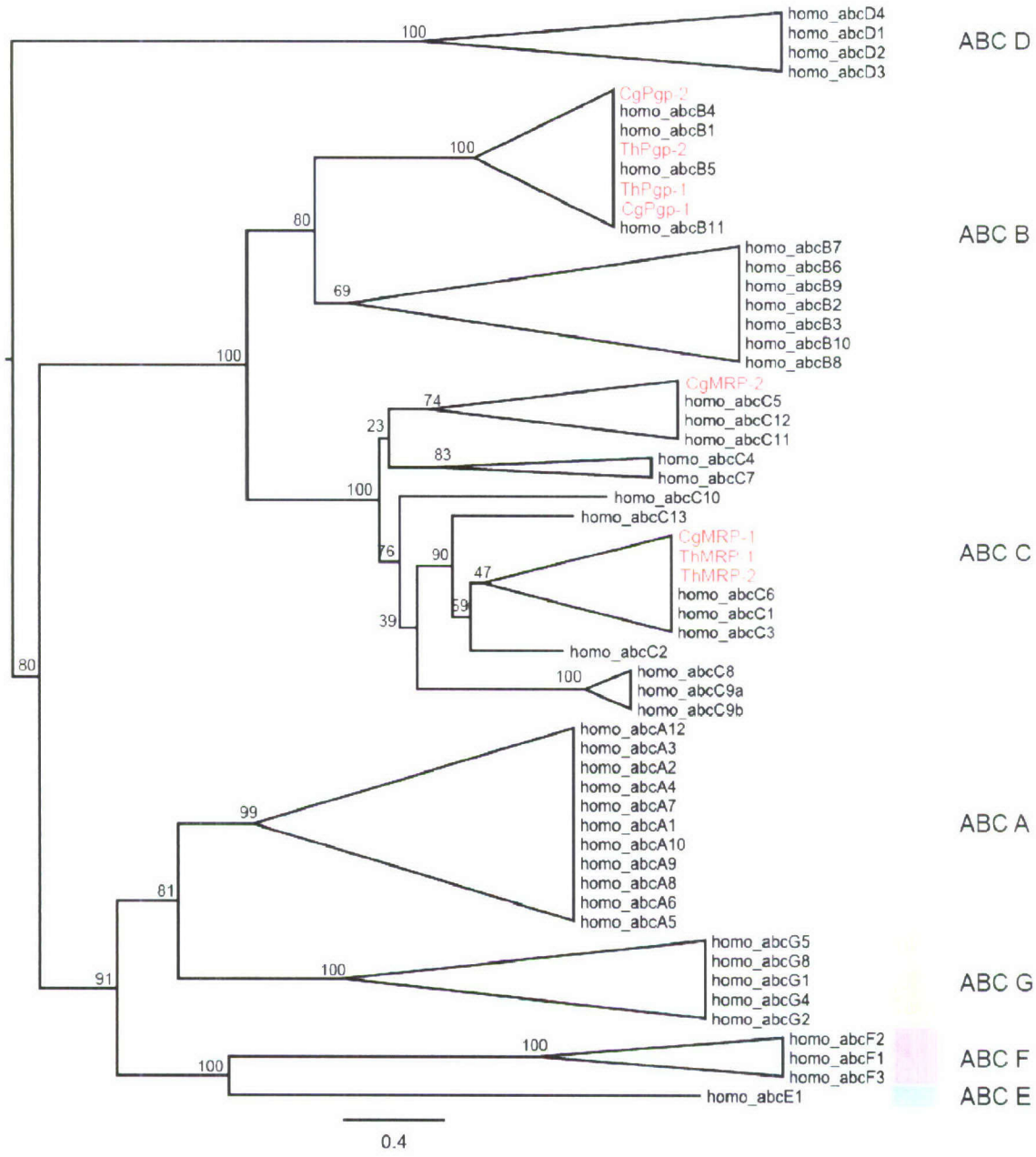


Figure 12. Comparison of molluscan and mouse Pgp hydropathy profiles. Deduced amino acid sequences of *Cyphoma* and *Tritonia* P-glycoproteins were aligned to mouse ABCB1a using ClustalX. The mouse ABCB1a plot is indicated by the red line and is overlaid by *Cyphoma* Pgp-1 (blue line), *Cyphoma* Pgp-2 (green line), *Tritonia* Pgp-1 (maroon line), and *Tritonia* Pgp-2 (black line). Black bars above the plots indicate positions of the two transmembrane regions (TM1, TM2) and nucleotide binding domains (NB1, NB2) in ABCB proteins. Values above and below the horizontal line indicate hydrophobic and hydrophilic regions, respectively. Hydrophobicity plots were generated using the Kyte-Doolittle algorithm (1982) with a window of 13 residues by BioEdit v7.0.5.2. GenBank sequences in the alignment include: mouse ABCB1a (*M. musculus*, NP_035206), *C. gibbosum* Pgp-1 (EU487190), *C. gibbosum* Pgp-2 (EU487191), *T. hamnerorum* Pgp-1 (EU487196), *T. hamnerorum* Pgp-2 (EU487197).

Figure 13. Phylogenetic relationships among *Cyphoma*, *Tritonia* and human ABC transporters. Maximum likelihood (ML) trees were constructed as described in the Methods. Out of a possible 3450 positions, 1821 positions (or 52.8%) were used to construct the final tree. Molluscan sequences are highlighted in red. Values at branch points represent ML bootstrap values calculated with 100 replications. Triangles represent portions of the tree that were collapsed due to poor resolution of the taxa within each clade as evidenced by bootstrap analysis. GenBank sequences in the tree include: Human ABCA proteins (ABCA1, NP_005493.2; ABCA2, NP_001597.2; ABCA3, NP001080.2; ABCA4, NP_000341.2; ABCA5, NP_061142.2; ABCA6, NP_525023.2; ABCA7, NP_061985.2; ABCA8, NP_009099.1; ABCA9, NP_525022.2; ABCA10, NP_525021.3; ABCA12, NP_775099.2), human ABCB proteins (ABCB1, NP_000918.2; ABCB2, NP_000584.2; ABCB3, NP_000535.3; ABCB4, NP_000434.1; ABCB5, NP_848654.3; ABCB6, NP_005680.1; ABCB7, NP_004290.2; ABCB8, NP_009119.2; ABCB9, NP_982269.1; ABCB10, NP_036221.1; ABCB11, NP_003733.2), human ABCC proteins (ABCC1, NP_004987.2; ABCC2, NP_000383.1; ABCC3, NP_003777.2; ABCC4, NP_005836.2; ABCC5, NP_005679.2; ABCC6, NP_001162.3; ABCC8, NP_000343.2; ABCC9 a/b, NP_005682.2, NP_064693.2; ABCC10, NP_258261.2; ABCC11, NP_115972.2; ABCC12, NP_150229.2; ABCC13, EAX10058.1), human ABCD (ABCD1, NP_000024.2; ABCD2, NP_005155.1; ABCD3, NP_002849.1, ABCD4, NP_005041.1), human ABCE (ABCE1, NP_002931.2), human ABCF (ABCF1, NP_001020262.1; ABCF2, NP_009120.1; ABCF3, NP_060828.2), human ABCG (ABCG1, NP_997057.1; ABCG2, NP_004818.2; ABCG4, NP_071452.2; ABCG5, NP_071881.1; ABCG8, NP_071882.1), *Cyphoma gibbosum* MRP-1 (EU487192), MRP-2 (EU487193), Pgp-1 (EU487190), Pgp-2 (EU487191), *Tritonia hamnerorum* MRP-1 (EU487194), MRP-2 (EU487195), Pgp-1 (EU487196), Pgp-2 (EU487197). Analysis performed by J. Goldstone.


homo_abcD4
homo_abcD1
homo_abcD2
homo_abcD3
ABC D
CgPgp-2
homo_abcB4
homo_abcB1
ThPgp-2
homo_abcB5
ThPgp-1
CgPgp-1
homo_abcB11
ABC B
homo_abcB7
homo_abcB6
homo_abcB9
homo_abcB2
homo_abcB3
homo_abcB10
homo_abcB8
CgMRP-2
homo_abcC5
homo_abcC12
homo_abcC11
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homo_abcC7
homo_abcC10
homo_abcC13
CgMRP-1
ThMRP-1
ThMRP-2
homo_abcC6
homo_abcC1
homo_abcC3
ABC C
homo_abcC2
homo_abcC8
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homo_abcA8
homo_abcA6
homo_abcA5
ABC A
homo_abcG5
homo_abcG8
homo_abcG1
homo_abcG4
homo_abcG2
ABC G
homo_abcF2
homo_abcF1
homo_abcF3
ABC F
homo_abcE1
ABC E
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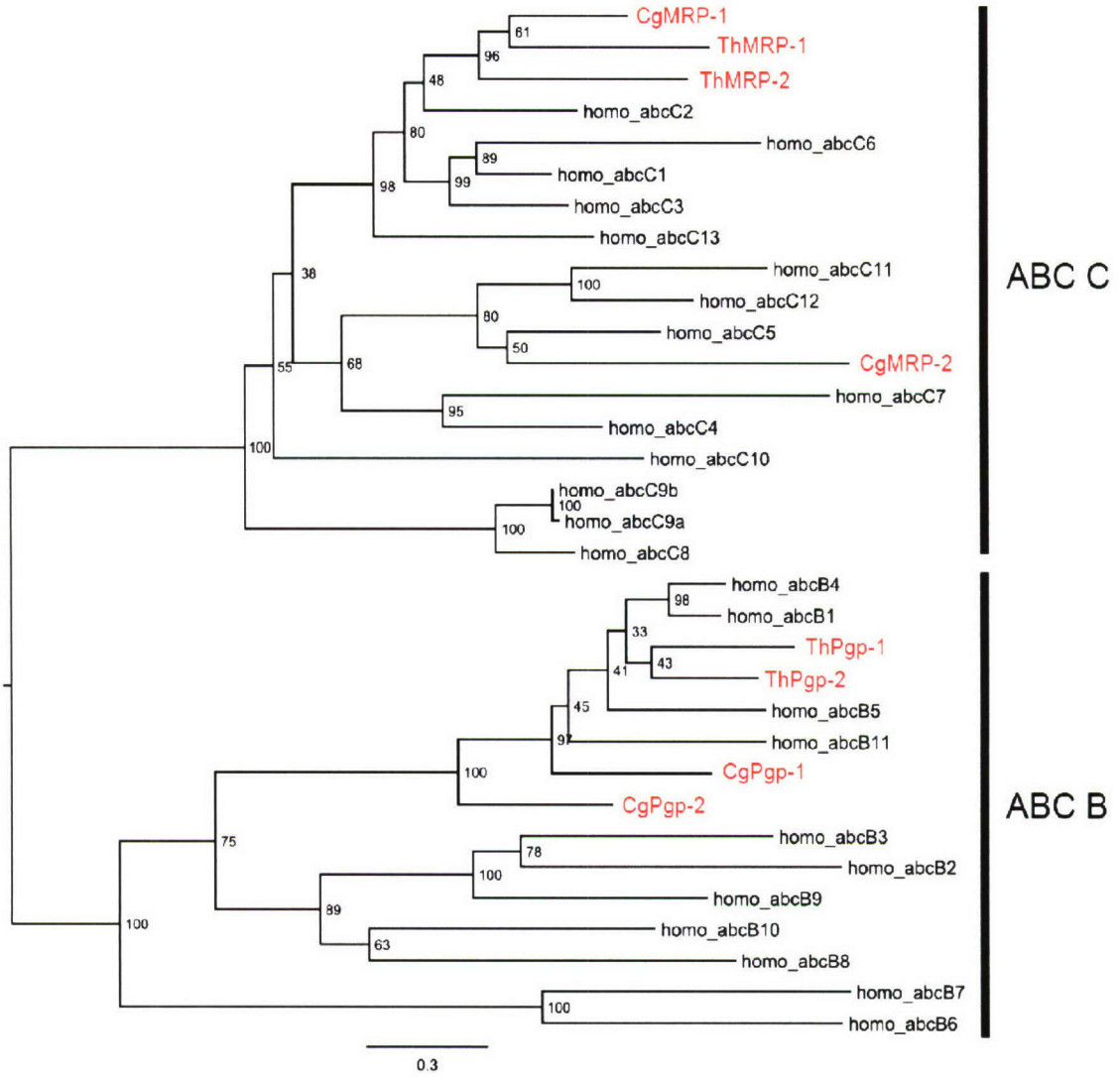


Figure 14. Phylogenetic relationships among *Cyphoma*, *Tritonia* and human ABCB and ABCC protein subfamilies. Maximum likelihood (ML) trees were constructed as described in the Methods. Out of a possible 2026 positions, 1402 positions (or 69.2%) were used to construct the final tree. Molluscan sequences are highlighted in red. Values at branch points represent ML bootstrap values calculated with 100 replications. GenBank sequences in the tree include: Human ABCB proteins (ABCB1, NP_000918.2; ABCB2, NP_000584.2; ABCB3, NP_000535.3; ABCB4, NP_000434.1; ABCB5, NP_848654.3; ABCB6, NP_005680.1; ABCB7, NP_004290.2; ABCB8, NP_009119.2; ABCB9, NP_982269.1; ABCB10, NP_036221.1; ABCB11, NP_003733.2), human ABCC proteins (ABCC1, NP_004987.2; ABCC2, NP_000383.1; ABCC3, NP_003777.2; ABCC4, NP_005836.2; ABCC5, NP_005679.2; ABCC6, NP_001162.3; ABCC8, NP_000343.2; ABCC9 a/b, NP_005682.2, NP_064693.2; ABCC10, NP_258261.2; ABCC11, NP_115972.2; ABCC12, NP_150229.2; ABCC13, EAX10058.1), *Cyphoma gibbosum* MRP-1 (EU487192), MRP-2 (EU487193), Pgp-1 (EU487190), Pgp-2 (EU487191), *Tritonia hamnerorum* MRP-1 (EU487194), MRP-2 (EU487195), Pgp-1 (EU487196), Pgp-2 (EU487197). Analysis performed by J. Goldstone.

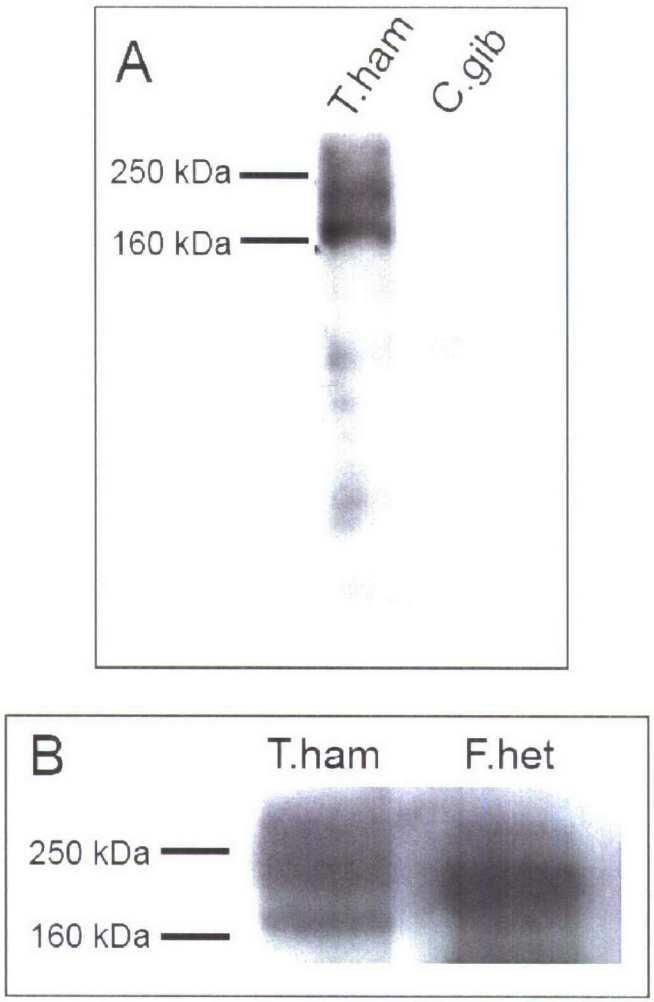


Figure 15. Detecting Pgp expression in molluscan tissue homogenates. Western blots were probed with anti-P-glycoprotein mAb C219 which reacted with (**A**) one sharp band at 170 kDa and two diffuse bands at > 170 kDa in *T. hamnerorum* whole cell homogenates. No immunopositive bands were detected in *C. gibbosum* digestive gland homogenates. (**B**) *Fundulus heteroclitus* liver cell lysates were used as a positive control.

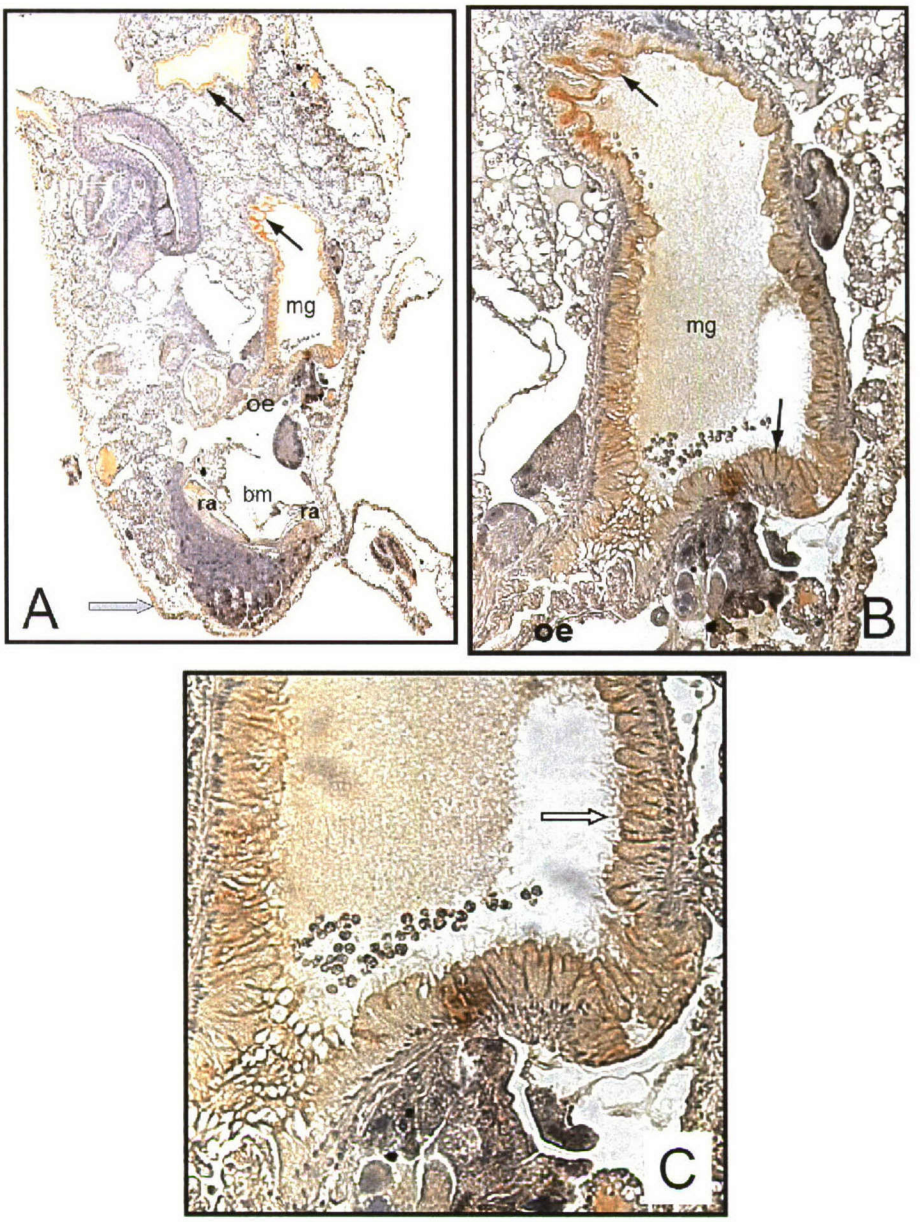


Figure 16. Immunolocalization of Pgp in *T. hamnerorum* tissues. Tangential sections of *T. hamnerorum* were probed with the anti-P-glycoprotein mAb C219. Orange-brown staining indicates immunoreactive protein. **(A)** Black arrows indicate intense staining by C219 along the apical tips of the gut epithelium; grey arrow indicates lighter staining by C219 along the epidermis; *bm*. buccal mass; *mg*. midgut; *oe*. esophagus; *ra*. radula; (50x). **(B)** Magnification (100x) of esophagus and midgut. **(C)** Further magnification (200x) of the ciliated columnar epithelia in the midgut; cilia indicated by white arrow.

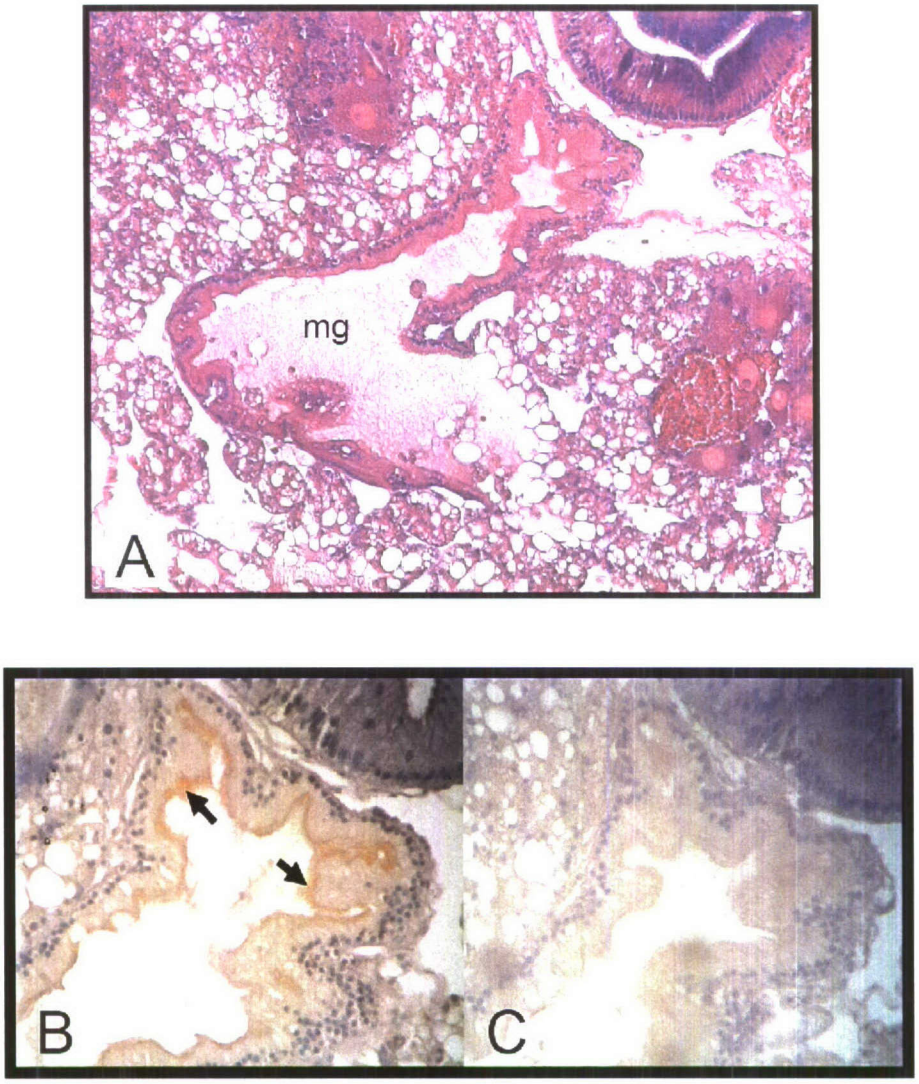


Figure 17. Immunolocalization of Pgp in *T. hamnerorum* tissues continued. (A) Hematoxylin and eosin stained section depicting the folded nature of *T. hamnerorum* midgut tissues; *mg*. midgut (100x). **(B)** C219 probed tissue section and **(C)** unlabeled serial section showing P-glycoprotein localized to the periphery of the midgut epithelium (indicated by black arrows) (200x)

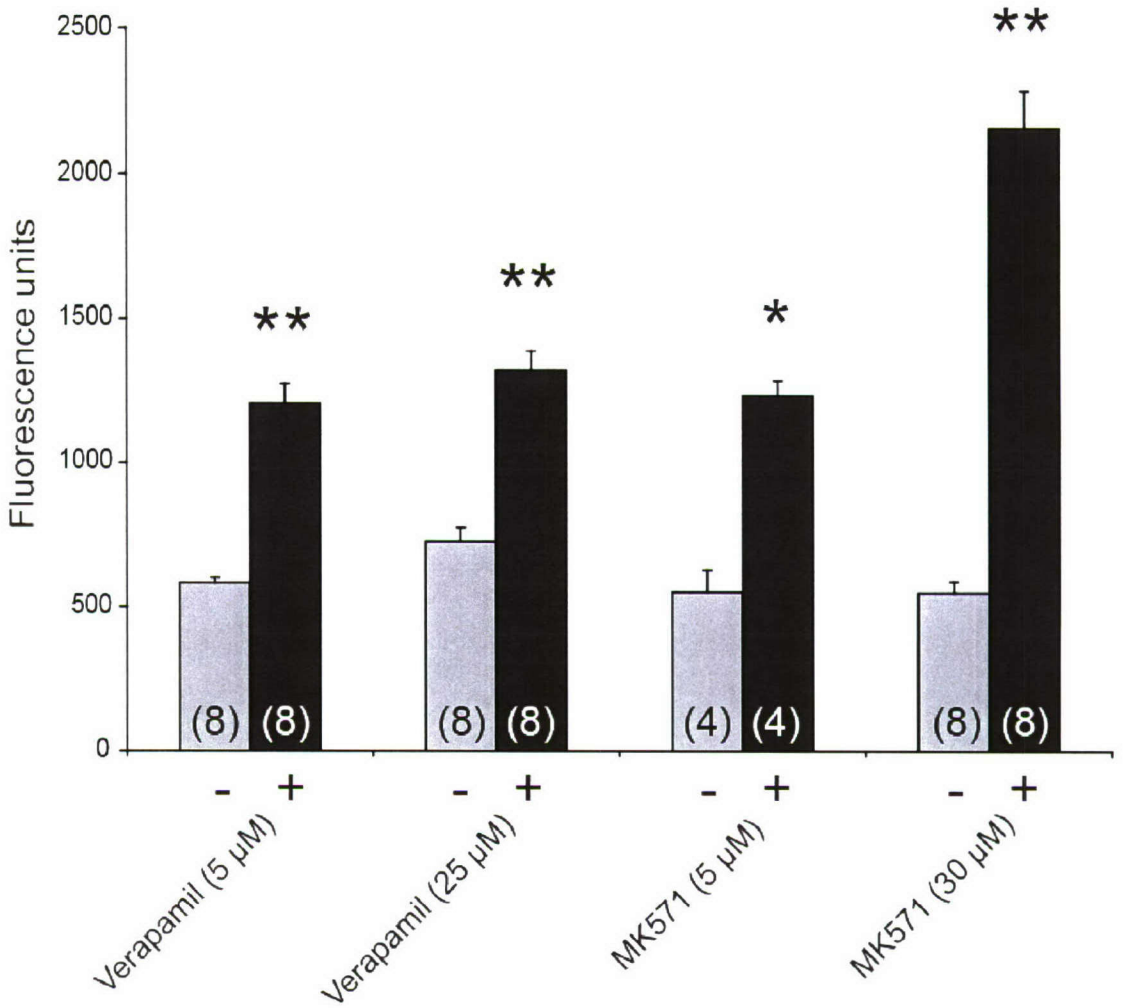


Figure 18. Change in intracellular fluorescence in *T. hamnerorum* incubated with MRP (MK571) and Pgp (verapamil) inhibitors compared to untreated controls. Bars represent mean fluorescence (± SE) of *T. hamnerorum* tissue homogenates from four or eight replicate wells (replicates indicated in parentheses). All inhibitor exposures resulted in a significant increase in calcein accumulation. MK571 at 30 µM exposure resulted in the greatest inhibition of transport activity in *T. hamnerorum*. (*p=0.001, **p<0.001, Two sample t test)

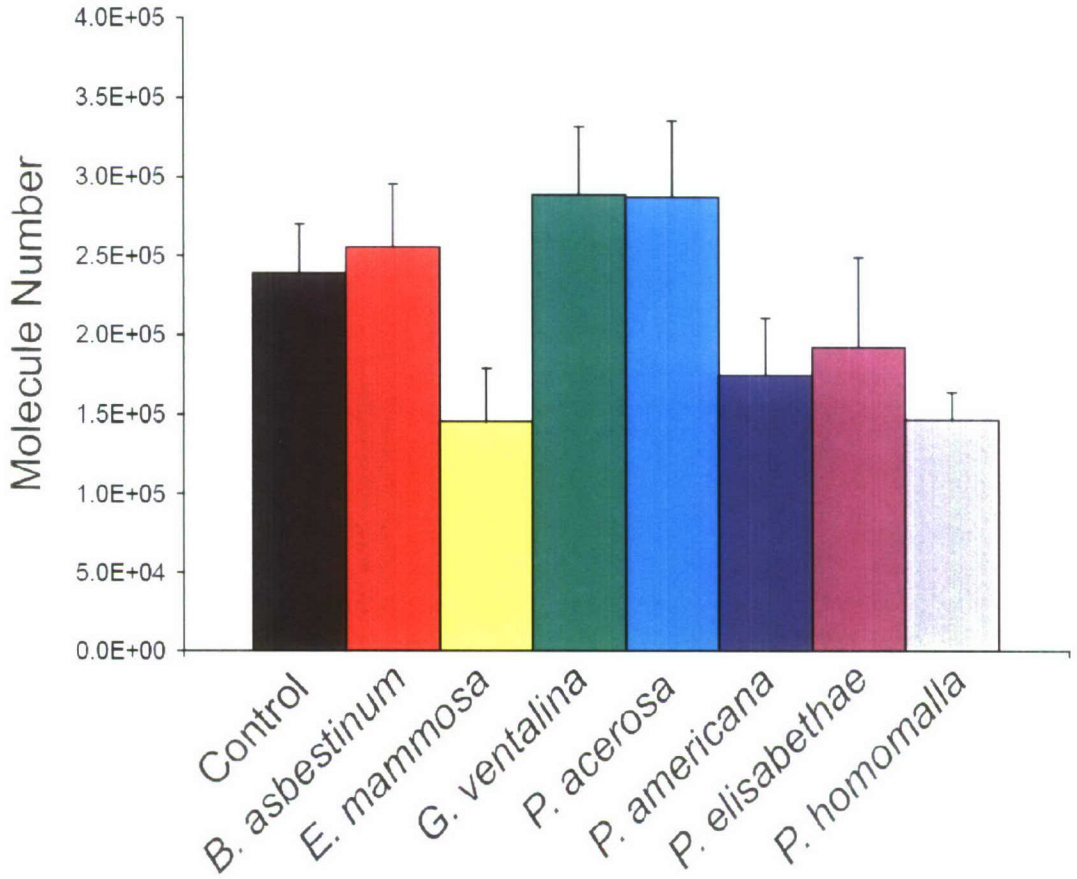


Figure 19. Mean MRP-1 transcript expression among *C. gibbosum* individuals feeding on a gorgonian or control diet for four days. Bars represent the mean MRP-1 transcript expression (± SE) of snails feeding on *B. asbestinum* (n = 13 snails), *E. mammosa* (n = 12), *G. ventalina* (n = 13), *P. acerosa* (n =10), *P. americana* (n = 12), *P. elisabethae* (n = 6), *P. homomalla* (n = 11) or a control diet (n = 33). Real-time quantitative PCR was performed and the number of transcript molecules per 0.2 μg of poly(A)+ RNA was calculated from the standard curve and normalized by a β-actin correction factor. Results of a two-way ANOVA indicate no significant differences in MRP-1 expression in snails feeding on different diets.

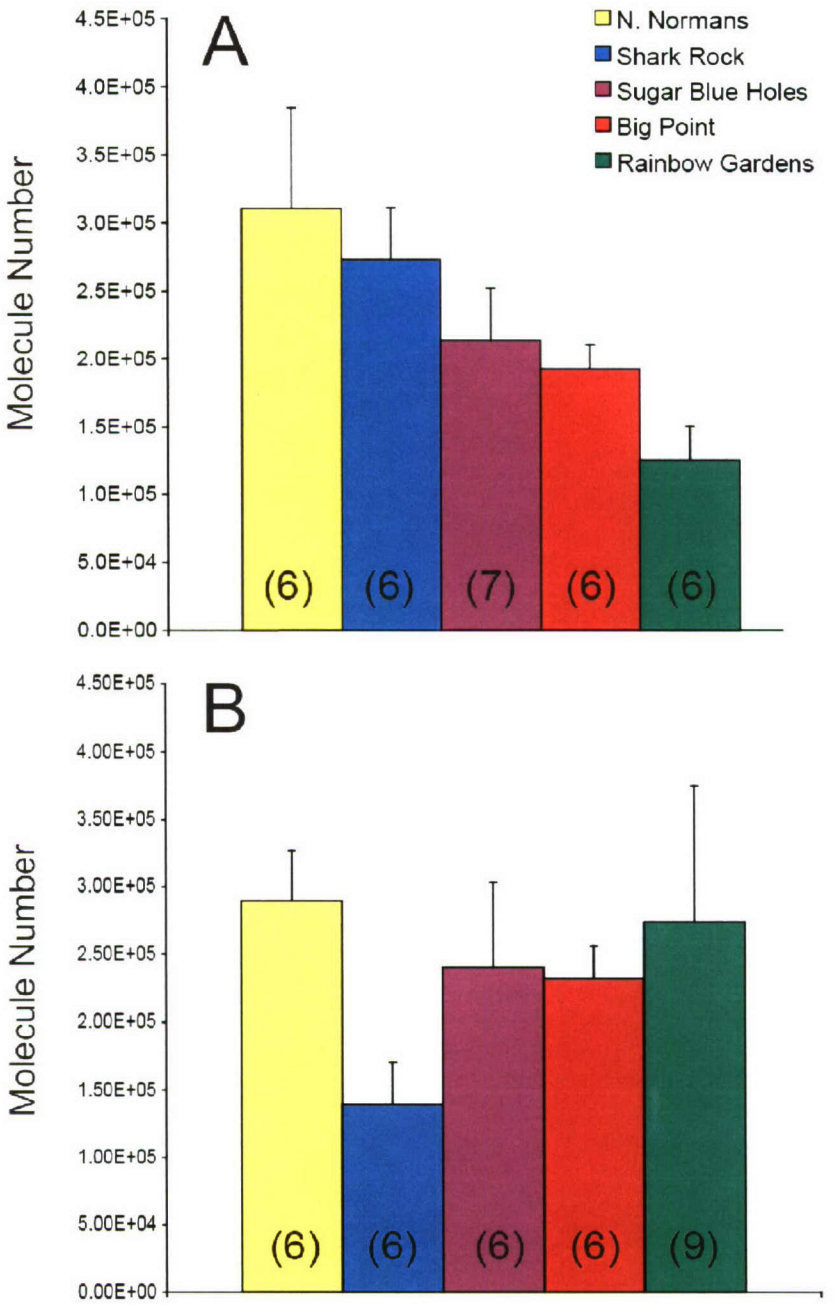


Figure 20. Mean MRP-1 transcript expression among time-zero and four day control diet fed *C. gibbosum* collected from five reefs. (**A**) Time-zero snails; mean MRP-1 transcript expression (± SE) in snails (n = 31 snails) collected from five reefs and immediately dissected to preserve reef-specific transcript expression signals. (**B**) Control snails; mean MRP-1 transcript expression (± SE) in snails (n = 33 snails) collected from five reefs and fed a control diet (e.g., alginic acid + squid powder) for four days. Values in parentheses indicate the number of replicate snails examined per reef. Real-time quantitative PCR was performed and the number of transcript molecules per 0.2 µg of poly(A)+ RNA was calculated from the standard curve and normalized by a β-actin correction factor.

Chapter VI

Conclusions and Future Directions

The marriage between molecular/proteomic tools and traditional methods for the study of marine consumer-prey interactions holds considerable promise for the field of chemical ecology. Despite the tremendous progress in identifying the diversity, concentrations and distribution of allelochemicals and their effects on the behavior of marine consumers, few studies have addressed the proximate mechanisms underlying the profound variation in tolerance among these consumers for their chemically defended foods. The work presented in this thesis demonstrates the power of emerging technologies available to chemical ecologists to characterize for the first time those biochemical innovations postulated to lead to the adaptation of marine consumers to their allelochemically-defended prey. Elucidation of consumer environmental response genes encoding proteins involved in the detoxification and transport of natural products will undoubtedly ignite exciting science at the interface of pharmacology and marine ecology. Moreover, identifying the molecular underpinnings of organismal physiological response has broad implications for understanding the role of the environment in determining gene function in a co-evolutionary context. What follows in this chapter is the summation of a multidisciplinary study investigating the expression, activity and diversity of three dominant families of enzymes involved in xenobiotic resistance, providing the most comprehensive assessment thus far of biochemical resistance mechanisms used by marine consumers to cope with their chemically defended prey.

A Proteomic Approach to Chemical Ecology

Glutathione *S*-transferases (GSTs) have long been known to play pivotal roles in xenobiotic resistance in insects and the high GST activity in the digestive gland of our model consumer, *Cyphoma gibbosum* (Vrolijk & Targett 1992), further indicated their potential importance in allelochemical detoxification. However, information as to the diversity, isoform-specific expression, and relatedness of *C. gibbosum* GSTs to those of metazoans was lacking. After a molecular approach with degenerate primers designed to previously cloned molluscan GSTs failed to yield any GST sequences, efforts were redirected toward a proteomic approach that proved to be highly successful, facilitating the identification of the collection of expressed GSTs in *C. gibbosum* digestive gland. Peptide fragment analysis revealed that both mu- and theta-class GSTs were expressed in molluscan tissues – the latter being the first theta-class GST identified from a molluscan source. Mu-class GSTs are known to mediate the conjugation of electrophilic natural products, including prostaglandins (previously characterized as gorgonian feeding deterrents), while theta-class GSTs are unique in their ability to act as dehalogenases and may protect *Cyphoma* from a host of organohalogens found in its gorgonian diet.

RT-PCR and RACE cloning efforts with degenerate primers designed to homologous peptides obtained from proteomic analyses yielded two full-length mu-class GST cDNA sequences from *C. gibbosum* digestive gland. Comparative and phylogenetic analysis revealed that *Cyphoma* GST mu homologs share 78% amino acid identity and form a monophyletic group with other molluscan GST mu sequences. Attempts were made to further biochemically characterize individual *Cyphoma* mu-class GST isoforms

by recombinant expression in yeast. While immunochemical analysis confirmed the expression of *Cyphoma* mu-class GSTs in yeast systems, GST activity was never observed with recombinant cytosolic fractions. It is possible the choice of expression system (yeast vs. *E. coli*) may have affected protein activity (e.g., inhibitor present), yet studies successfully expressing GSTs for functional characterization in both systems have been reported. Additional possibilities include the prevention of GST homodimer formation due to the presence of the C-terminal epitope tag used to visualize protein expression. Alternatively, *Cyphoma* GSTs may function as heterodimers, not uncommon among mu-class GSTs, and co-expression of both cDNAs would be necessary for GST activity to be restored. The potential of GST subunits to form heterodimers would likely increase the pool of potential substrates available for conjugation. Future optimization of recombinant *Cyphoma* GST expression systems will allow for further biochemical characterization of paralogous GST isoforms and permit the testing of hypotheses concerning possible subfunctionalization of specific GST isoforms following gene duplication and divergence events.

Cyphoma GSTs: the role of constitutive biochemical defense mechanisms

In contrast to most housekeeping genes, genes involved in xenobiotic metabolism often are strongly inducible by substrates upon which they act, accelerating the biotransformation and elimination of the substrate. Interestingly, *Cyphoma* digestive gland tissues constitutively express high levels of GST regardless of the diet of this consumer. Further, separation of *Cyphoma* GST proteins by HPLC revealed that GST

subunits are expressed in the digestive gland in the same relative abundance in individuals feeding on different diets. These findings led me to test the hypothesis that the constitutive expression of GSTs may be advantageous to our generalist if the majority of gorgonian diets contain substrates for *Cyphoma* GSTs. A screen of crude extracts from eight species of gorgonian corals, commonly serving as hosts for *Cyphoma* in the field indicated that all extracts contained potent inhibitors of these enzymes, the most inhibitory of which were traced to the moderately hydrophobic, chloroform-soluble fractions. The sensitivity of *Cyphoma* GSTs to inhibition by these extracts suggests that gorgonian diets contain electrophilic substrates or inhibitors. GSTs are most notable for their ability to conjugate electrophilic substrates with glutathione; however, the ability of these enzymes to act as 'xenobiotic sponges' by sequestering non-substrate ligands (Ketley et al. 1975, Kostaropoulos et al. 2001) may serve as further protection against allelochemical toxicity. Additionally, the presence of inhibitors could represent counter-defense strategies employed by gorgonian hosts to block the GST-mediated metabolism of co-occurring allelochemicals by *Cyphoma*. Further studies at both the biochemical and organismal level would be needed to determine if potent gorgonian inhibitors could reduce the effectiveness of *Cyphoma* GSTs and thereby enhance toxicity.

Specific gorgonian compounds known for their ecological and biological activities were investigated for their ability to serve as substrates for *Cyphoma* GSTs. In mammals, prostaglandins are hormone-like fatty acids that exert their effects through G-protein-coupled receptors and influence a variety of cellular processes including induction of apoptosis, inhibition of cell growth, activation of oxidative stress pathways

and inflammation response. In marine systems, selected prostaglandin series (PGA_2, PGE_2 and $PGF_{2\alpha}$) are found in unusually high concentrations in gorgonian tissue, where they function as antipredatory compounds. Some studies report that *Cyphoma* seems unaffected by the prostaglandins in its diet, preferring in some localities to reside on gorgonian species (e.g., *Plexaura* sp.) known to harbor high concentrations of prostaglandins. A screen of commercially available prostaglandins revealed that those in the A series, which are most abundant in gorgonians, were the most potent inhibitors of *Cyphoma* GST activity. Kinetic studies with affinity-purified GSTs indicated that 15(S)-PGA_2 was a competitive inhibitor, and likely a substrate for *Cyphoma* GSTs, with apparent affinity values comparable to K_m constants described for vertebrate prostaglandin-conjugating GSTs. Estimates of the *in vivo* prostaglandin concentration experienced by *Cyphoma* when foraging suggests that GSTs would be saturated with respect to the PGA_2 and would be operating near the enzyme's physiological limits. However, the prostaglandin-conjugating ability of *Cyphoma* GSTs will need to be substantiated in incubation experiments by demonstrating the formation of prostaglandin-glutathione conjugates above non-enzymatic levels.

The results of Chapter 4 indicate that high, constitutive activity of *Cyphoma gibbosum* GSTs is likely necessitated by the ubiquitous presence of substrates/inhibitors in this consumer's gorgonian diet. Evidence from terrestrial insect-host studies also indicate that generalist counter-defense enzymes often have a broader substrate specificity than those of comparable specialists. The greater functional versatility of detoxification enzymes in generalists may facilitate the exploitation of a greater range of

chemically diverse diets. Therefore, it would be interesting to compare GST expression and substrate metabolism profiles between the generalist *Cyphoma gibbosum* and the sympatric specialist *Cyphoma signatum*, which feeds solely on a single gorgonian species. It is possible that the success of the polyphagous foraging strategy of *C. gibbosum* is due to the constitutive expression of catalytically versatile GSTs that allow this consumer to cope with its chemically unpredictable prey.

Cyphoma P450s: gene specific induction in response to select suites of gorgonian allelochemicals

The majority of previous research assessing the response of marine consumer detoxification enzymes to allelochemical exposure has relied upon the use of enzymatic activity with diagnostic substrates or immunochemical detection with vertebrate-derived probes. While these methods are a good first step, often the selection of probes or diagnostic substrates has been biased toward vertebrate gene families or subfamilies involved in pharmacological or pollutant response, which may have little or no ecological relevance to allelochemical metabolism in the organism being studied. A more quantitative and sensitive approach to examine the tissue-specific level of putative allelochemical detoxification proteins involves quantitative PCR, which takes advantage of available molecular evidence and does not require first hand knowledge of protein function.

Cyphoma cytochrome P450 Family 4 (CYP4) members were originally targeted for expression analysis based on previous evidence in other organisms suggesting that

classes of compounds analogous to those found in gorgonian tissues could be inducers and substrates of CYP4 proteins. Using molecular techniques, I identified several distinct CYP4 genes from *Cyphoma* digestive gland. Analysis of *Cyphoma* feeding on different diets revealed that mRNAs encoding specific CYP4 enzymes were regulated in a diet-specific manner. Select CYP4 transcripts were significantly induced in snails feeding on *Plexaura homomalla*, a gorgonian diet known to contain high concentrations of prostaglandins. Phylogenetic analysis of allelochemically-responsive molluscan CYP4 cDNAs indicated that the closest vertebrate relatives were those of the fatty acid metabolizing CYP4A and CYP4F subfamilies, both of which contain members with prostaglandin hydroxylase activity. A comparison of molluscan CYP4 genes with vertebrate fatty acid hydroxylase members revealed that molluscan enzymes share key amino acid residues within their substrate access channel and active site known to mediate prostaglandin hydroxylase activity in vertebrates. Heterologous expression of selected cDNAs indicated a possible role of *Cyphoma* CYP4s in eicosanoid metabolism. Furthermore, microsomal protein isolated from the digestive gland of snails feeding on *P. homomlla* had a greater propensity to metabolize the diagnostic fatty acid, leukotriene B_4, in comparison to microsomes isolated from control snails. The increased eicosanoid metabolism in *P. homomalla* fed snails is consistent with the earlier observation that selected CYP4 enzymes are induced in snails feeding on this particular gorgonian diet, likely in response to the high concentrations of dietary prostaglandins.

The weight of evidence for *Cyphoma* CYP4s as prostaglandin metabolizing genes is striking. The next obvious step is to examine the metabolism of ecologically relevant

prostaglandins by recombinant *Cyphoma* CYP4s. In addition, feeding assays with purified gorgonian prostaglandins incorporated into artificial diets offer a way to assess the rate and specificity of *Cyphoma* CYP4 transcript induction in response to specific allelochemical stressors. These additional studies will allow for a more complete description of the molecular and biochemical pathways likely responsible for mediating dietary prostaglandin tolerance in *Cyphoma*.

The results of this chapter have built a firm foundation from which to test additional hypotheses about the evolution and transcriptional regulation of environmental response genes in marine consumers. Molecular characterization of CYP4 transcripts expressed in *C. gibbosum* indicates that allelochemical-responsive CYP4 subfamilies have undergone extensive radiation in this generalist, likely driven in part by co-evolutionary processes unique to the natural history of this consumer. Sequence variations have accrued in the coding regions, including substrate recognition sites, of these genes and this would suggest that despite their high degree of sequence similarity, these proteins may have significantly different metabolic capabilities and/or substrate specificities. The sequence diversity of allelochemically-responsive *Cyphoma* CYP4 subfamily members may reflect the enzymatic diversity required for generalists to metabolize the range of prostaglandin analogs in gorgonian tissues (PGA_2, PGE_2 and $PGF_{2\alpha}$). Heterologous expression of polymorphic transcripts and subsequent analysis of prostaglandin metabolism would help define the key residue(s) required for metabolism of specific prostaglandin classes.

In addition, promoter analysis of allelochemically-responsive CYP4 members is likely to identify a number of putative *cis*-acting transcriptional response elements that are possible targets of regulation by gorgonian allelochemicals. Among the ligand-activated nuclear transcription factors known to regulate both CYP4 and GST expression are those in the peroxisome proliferator-activated receptor (PPAR) family, known for their roles in homeostasis, inflammation and wound repair. Cyclopentenone prostaglandins, including PGA_2 series, are among the most potent of the natural PPAR agonists and it is likely that PPAR or related transcription factors orchestrate the induction of a battery of detoxification genes in *Cyphoma* in response to dietary prostaglandins. Sequencing of the sea urchin genome revealed two PPAR paralogs are present in invertebrate deuterostomes (Goldstone et al. 2006); however, little information exists as to the true number of nuclear receptors and their functions in molluscan species. Future work examining the ability of ecologically relevant prostaglandins to activate putative *Cyphoma* PPARs or related orphan nuclear receptors in cell-based reporter gene assays will help elucidate the molecular pathways responsible for gorgonian allelochemical detoxification. In sum, the work described in this chapter provides a framework for a more mechanistic understanding of the regulatory pathways mediating the expression of allelochemical metabolizing genes.

Possible roles of efflux transporters as consumer counter-mechanisms to prey chemical defenses

Multixenobiotic resistance transporters (MXRs) are often considered an organism's first line of defense against environmental chemicals, yet they are frequently overlooked as possible allelochemical resistance mechanisms by many in the chemical ecology field. The multixenobiotic efflux phenotype has been described in several aquatic organisms, and numerous natural products - including marine examples - have been shown to inhibit the efflux of probe substrates for these transporters. The wide taxonomic distribution and broad substrate specificity of these transporters led me to hypothesize that MXRs may aid marine consumers in coping with dietary allelochemical stress. In addition to their efflux capabilities, MXRs have also been shown to facilitate the intracellular concentration of substrates in subcellular vesicles or organelles as another possible mechanism of reducing intracellular toxicity (Van Luyn et al. 1998, Molinari et al. 2002, Rajagopal & Simon 2003, Ifergan et al. 2005). Some have suggested that the ability of specialist consumers to concentrate the dietary chemical defenses of their prey or host for their own protection may be facilitated by MXR-mediated transport (Sorensen & Dearing 2006). However, no specific transporters have yet been implicated in allelochemical sequestration. The objective of Chapter 5 was to improve our understanding of the diversity of putative allelochemical efflux transporters, their activity, and their expression in both generalist (*Cyphoma gibbosum*) and specialist (*Tritonia hamnerorum*) molluscan predators that are likely to process dietary allelochemicals in different ways based on the needs of each consumer.

Molecular approaches yielded, from each molluscan species, four partial MXR fragments with homology to both human ABCB and ABCC subfamilies. Immunochemical analysis with a monoclonal antibody probe to P-glycoprotein (Pgp; ABCB1) detected immunoreactive proteins only in the tissue homogenates of *Tritonia* and not in the digestive gland homogenates of *Cyphoma*. *Tritonia* Pgp proteins were found to be associated with the apical tips of epithelia cells lining the gut lumen, a location consistent with a role in mediating dietary xenobiotic efflux. However, the lack of Pgp expression in *Cyphoma* homogenates and tissue sections was more ambiguous. Further inspection of partial deduced amino acid sequences of *Cyphoma* Pgps indicated that the disparity in protein expression may be due to lack of epitope recognition by the Pgp probe. These findings highlight the need for caution in interpreting immunochemical staining when probes developed in distantly related organisms are used.

Quantitative PCR is a more robust and sensitive approach for quantifying gene-specific expression and, consequently, was used to investigate the influence of gorgonian diet on MXR expression in *Cyphoma*. I initially targeted *Cyphoma* multidrug resistance protein (MRP-1) because of its putative role in the ATP-dependent export of glutathione (GSH) conjugates, based on homology to known GSH-X efflux transporters. Quantitative PCR analysis revealed that MRP-1 was constitutively expressed in snails feeding on all gorgonian diets. This result coupled with the findings in Chapters 2 and 4 suggest that *Cyphoma* GSTs may play a significant role in the detoxification of hydrophobic gorgonian allelochemicals and resultant allelochemical glutathione conjugates could potentially be substrates for *Cyphoma* MRP-1 proteins.

The *in vivo* activity of molluscan MXR transporters was assessed using a simple competitive dye efflux assay whereby MXR-specific inhibitors resulted in dye accumulation, implying the presence of MXR-mediated transport activity in *Tritonia* tissues. One question that was not addressed in studies in Chapter 5 was the ability of molluscan MXRs to transport (or sequester) gorgonian compounds. In the future, these same dye assays can be used to screen gorgonian extracts and pure compounds of interest using a bioassay-guided fractionation approach to identify substrates/inhibitors for molluscan MXR transporters both in *in vivo* and *in vitro* with selected heterologously expressed molluscan proteins. This screening approach can also be used to test hypotheses about the substrate flexibility of generalist MXRs that may allow these consumers to export a greater diversity of compounds, reflective of the chemically diverse and unpredictable diets they encounter, in comparison to specialist consumers.

Future work: genomic approaches to understanding allelochemical modes of action and consumer counter-defenses

The field of chemical ecology has entered a new era whereby advances in molecular biology are set to provide physiological ecologists with the most comprehensive view of how cells and ultimately organisms tolerate and respond to allelochemical stresses. Techniques such as transcriptome profiling using DNA microarrays will allow for the parallel analysis of the expression of thousands of genes, providing a complete picture of gene regulation occurring in response to dietary compounds. This method does not limit the researcher to simple single gene hypotheses

rather, transcriptome profiling can be used to form *a posteriori* hypotheses about the mode of action of allelochemicals and consumer mechanisms of toxin resistance. The availability of genomic data from model marine organisms (e.g., anemone, sea urchin, sponge, oyster, limpet, polychaete, and tunicate) is rapidly expanding, and can be leveraged to investigate how small molecules can tranduce their effects through the genotype of an organism, ultimately affecting the phenotype and organism function. I am excited for the future of this field and the promise that genomic-enabled technology holds for rapidly advancing our understanding of allelochemical tolerance. Knowledge of how consumers respond and adapt to environmental chemical stressors on the molecular level will add volumes to our understanding of patterns of predation and herbivory in the marine environment.

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16. Abstract (Limit: 200 words)
The processes underlying dietary allelochemical tolerance are likely mediated, in part, by biochemical resistance mechanisms that have evolved under the selective pressure of host chemical defenses. Although many have invoked this hypothesis to explain the variation in marine consumer tolerance, few studies have examined the enzymatic diversity and corresponding metabolism of dietary allelochemicals.

Molecular and proteomic techniques identified both allelochemically-responsive cytochrome P450s (CYPs), and constitutively expressed glutathione *S*-transferases (GSTs) and ABC transporters in *Cyphoma gibbosum* digestive glands. Inhibition of *Cyphoma* GST activity by gorgonian extracts and selected allelochemicals (i.e., prostaglandins) indicated that gorgonian diets contain substrates for molluscan detoxification enzymes. Enzymatic studies with snail microsomes and recombinant CYPs suggested those *Cyphoma* enzymes most closely related to vertebrate fatty acid hydroxylating enzymes may contribute to the detoxification of ichthyodeterrent cyclopentenone prostaglandins found in abundance in selected gorgonian species. Finally, the presence and activity of multixenobiotic resistance transporters in *Cyphoma* and the co-occuring specialist nudibranch, *Tritonia hamnerorum*, suggests these efflux transporters could function as a first line of defense against dietary intoxication. Together, these results suggest marine consumers that regularly exploit allelochemical-rich prey have evolved both general (GST and ABC transporters) and allelochemical-specific (CYP) detoxification mechanisms to tolerate prey chemical defenses.

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